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Chemical Inhibitors for Biomass Yield Reduction in Activated Sludge

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Supervisor: Prof. Tom Stephenson

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ABSTRACT

Increasing legislation and rising treatment and disposal costs have promoted optimisation of the activated sludge process to encompass reduction of waste biomass. Manipulation of process control such as increasing sludge age and decreasing food to microorganism ratio can lower waste sludge production, but capital works as well as increased operating costs in the form of power requirement for oxygen supply may be required. The need for a cost effective method of biomass reduction without capital expenditure has prompted research into methods beyond process control. The use of chemicals capable of disrupting microorganism metabolic pathways can theoretically allow continuation of catabolic (degradative) paths whilst halting some or all of the anabolic (growth) pathways. This project explored the use of metabolic inhibitors (uncouplers, tricarboxylic acid cycle inhibitors and antibiotics) to reduce the yield of the activated sludge process. Initial respirometric studies identified many chemicals capable of interacting with the activated sludge microorganisms. Increased oxygen uptake rate was taken as an indication of a good uncoupler, and tests highlighted 4 chemicals with significant potential for achieving biomass reduction (trypan blue, rotenone, 2,4 DNP and 4 NP). These chemicals were then tested at a laboratory scale and at bench scale in both batch and continuous simulations. Simulations were carried out using activated sludge and settled sewage feed to obtain as realistic conditions as possible. In batch tests, trypan blue, rotenone and 2,4 DNP successfully reduced mixed liquor suspended solids accumulation with little effect on COD removal compared to controls. In continuous simulations, 2,4 DNP and 4 NP both lowered yield with respect to their relative controls. Rotenone addition did not result in lowered yield. In all cases, any yield reduction was not at the expense of process efficiency in terms of COD and BOD removal. At pilot scale, 2,4 DNP almost halved the observed yield compared to the control whilst having no significant effect on BOD, COD or ammonia removal, nitrite and nitrate production, SVI or CST. Addition of chemical uncouplers had little effect on the species diversity of the activated sludge though a reduction in the floc size was observed in treated samples. Selection of a suitable chemical can result in reduced yield without detrimental effect to process efficiency in the activated sludge process. An increase in oxygen consumption occurred which has an associated cost implication, but this was not found to be significant compared to the savings made by reducing the yield.

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And finally to the A. A. Milne character Winnie-the-Pooh, whose simple attitude to life helped put things into perspective. After an 'experiment' with a tree full of bees went rather wrong Winnie-the-Pooh crawled out of a gorse bush and muttered 'Oh bother', brushed the prickles off his nose and began to think again. 'Think, think, think!' he said, tapping his brow thoughtfully. 'Mmmmm time for a little smackeral of something' concluded Pooh, rubbing his tummy! (Winnie-the-Pooh, A. A. Milne)

ABBREVIATIONS

2,4 DNP	2,4 dinitrophenol
4 NP	4 nitrophenol
ATP	adenosine triphosphate
BAF	biological aerated filter
BOD	biological oxygen demand
CO_2	carbon dioxide
COD	chemical oxygen demand
CST	capillary suction time
EC	European community
EU	European Union
F:M	food to microorganism ratio or rate (kg BOD kg $MLSS^{-1}$ d^{-1})
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
NAD	nicatinaminde adenine dinucleotide
NADH	nicatinamide adenine dinucleotide hydrogenase
NH_3	ammonia
NO_2	nitrite
NO_3	nitrate
O_2	oxygen
OSA	oxic-settling-aerobic activated sludge system
Q	flow
RNA	riobonucleic acid
RPM	revolutions per minute
SD	standard deviation
SE	standard error
SVI	sludge volume index
TCA cycle	triccarboxylic acid cycle
UK	United Kingdom
USA	United States of America
UWWTD	Urban Wastewater Treatment Directive
Y_{BOD}	yield expressed in terms of BOD removal
Y_{COD}	yield expressed in terms of COD removal
Y_{OBS}	observed yield

NOTATION

[S]	concentration of substrate (mg ml^{-1})
K_I	inhibition constant (mg ml^{-1})
P_x	net waste activated sludge produced each day (kg d^{-1})
Q	influent flow (l d^{-1})
Q_e	effluent flow (l d^{-1})
Q_w	flow of waste sludge (l d^{-1})
S	average effluent BOD (mg l^{-1})
S_0	average influent BOD (mg l^{-1})
V	velocity of reaction (min^{-1})
V_{max}	maximum reaction velocity (min^{-1})
X	activated sludge MLSS (mg l^{-1})
X_e	effluent suspended solids (mg l^{-1})
X_w	MLSS of waste sludge (mg l^{-1})

Chapter 1: Introduction

Chapter 1

Introduction

Production of waste biomass in the form of sludge or slurry occurs as a result of biological wastewater treatment. Optimisation of existing technology and development of new techniques have been vital in order to meet the strict demands of the effluent consents, whilst treating increasing volumes of wastewater. The Urban Wastewater Treatment Directive (UWWTD) has imposed legislation that requires certain levels of treatment. Further European Union (EU) directives are in place to implement the criteria that influence how and where waste sludge may be disposed (Davis, 1996). A large proportion of UK waste sludge was disposed of at sea, however, EU legislation prohibited any discharge of sewage sludge to water bodies after the end of 1998 (CEC, 1991). Research has been directed towards finding suitable mechanisms for disposal to include considerations of sludge quality, costs and environmental impact. Waste sludge treatment and disposal is an environmentally sensitive and economically important issue.

Present sludge disposal options include recycling to agricultural land, land restoration, novel uses, incineration, landfill and coastal dumping (Hall, 1996). These options are listed in order of EC preference of use (Matthews, 1996). Sludge contains nutrients such as nitrogen, phosphate and organic matter and can be used to help enrich deficient soils or as a supplement to fertiliser. UK regulations to enforce EU legislation impose constraints to ensure high levels of heavy metals and pathogens are eliminated before sewage sludge may be applied to agricultural land (Bruce *et al.*, 1990; DOE, 1988). Sludge needs further treatment before disposal to land and this may not then be the cheapest or simplest route for disposal. Application to grassland, which is available all year when other agricultural land may not be, is another possibility for sludge disposal. The sludge still requires treatment to meet the standards of the EU directive (Hall, 1996).

Sludge can be utilised in land restoration to restore vegetation to areas without an established soil cover. The nutrient and organic matter in sludge compliment the deficiencies of such soils (Gray, 1989). Novel uses include low temperature pyrolysis to create fuel and production of building materials utilising waste sludge, but these are minor outlets compared to the volume of sludge requiring disposal (Hudson and Lowe, 1994). Although the proportion of waste sludge incinerated will rise with the prohibition of coastal dumping, problems with public perception of facilities will cause difficulties (Hall, 1996).

Generally, incineration units are unwelcome in close proximity to urban areas due to public fears about emissions, this also creates problems in obtaining planning permission (Hall, 1996). Many advances have been made in incineration technology in terms of reduction and control of gas emissions to meet strict standards. Incineration results in about 30 % ash, which requires disposal, usually in landfill. Landfill is only a short term option as reducing availability increases costs; 40 % of sewage sludge is landfilled in Europe compared to 8 % in the UK (Hall, 1996). Problems of stability and in handling are encountered due to the poor physical properties of sludge.

Economical and safe sludge disposal will always play a vital part of wastewater treatment; with the diminishing disposal outlets reduction of sludge at source becomes increasingly important. Reduction to almost zero solids is possible by wet oxidation of solids. These systems involve destructive techniques including thermal degradation, hydrolysis and oxidation of organic matter in sludge in a single reactor. Sub critical conditions for this have been achieved by VerTech in the Netherlands, using deep well technology (Hall, 1996; Hudson and Lowe, 1994). A full scale plant has been operated in Holland at Apeldorn that achieved 70 % COD reduction across the reactor and temperatures of 270°C were achieved at the base of the reactor. The plant consisted of a cement encased well 1200m deep and 95 cm in diameter in which concentric tubes were suspended together with heat exchangers for temperature control. Despite resulting in very little sludge, the high costs and practical considerations make the system impractical for general use. The possibility of widespread implementation of

such a system is unlikely and the consequential impact on UK waste sludge production will be limited.

Sludge generated by wastewater treatment processes arises from several sources: primary sludge, waste or surplus activated sludge and humus sludge. The sludge arising from secondary treatment processes is often recirculated to the primary sedimentation tanks and cosettled with the primary sludge. Sludges contain varying amounts of nitrogen, phosphorus and other nutrients (Table 1.1). The total dry solids content of sludge will alter according to the treatment of the sludge. Untreated liquid sludge has the lowest percentage dry solids (1.5 – 6.0 %), dewatering of the sludge can increase the dry solids content to 25 % (CIWEM, 1995).

Table 1.1: Typical nutrient and solids contents of sewage sludges (Nitrogen, phosphorus and potassium as % on dry solids, dry solids and organic matter as % of total wet weight) (adapted from CIWEM, 1995).

Type of sludge	Total nitrogen	Soluble nitrogen	Phosphorus (as P)	Potassium (as K)	Total dry solids	Organic matter
Primary	2.5	0.1	1.0	0.2	6.0	4.2
Surplus activated	5.0	0.2	0.6	<0.05	1.5	1.2
Humus	4.0	0.2	0.6	<0.05	2.5	1.8
Primary and secondary combined	3.5	0.2	1.3	0.2	4.5	3.4

The UWWTD requires effluents from all wastewater treatment works with a population equivalent of more than 2000 to be treated according to the sensitivity of the receiving water (CEC, 1991). This has resulted in many treatment works increasing the level of treatment carried out, which often results in increased volumes of sludge which also need treatment and disposal. The UK produced 1019 x 10^3 tonnes

of sludge in 1993, this figure is set to rise to around 1602 x 10^3 in the year 2000 (Davis, 1996). This is a vast increase in the amount of sludge requiring disposal. This increase in sludge will occur mostly due to improved treatment facilities to accommodate the more stringent discharge consents imposed by the UWWTD. However, new sewage treatment facilities (generally at coastal locations) required due to the cessation of coastal sludge dumping will also contribute, along with population growth (Brown and Whipps, 1994). Any process that can aid the reduction of waste sludge output will help ease pressure on the outlets for disposal and lower costs.

The microbial cells in biological processes use external substrate (wastewater) in many metabolic paths; some which result in growth, production of new cells and product synthesis and others that generate energy for maintenance reactions. The extent to which substrate is converted to products or biomass is expressed as the yield. There are many definitions of yield in biological processes; the observed or apparent yield (Y_{obs}) is defined as the mass (or moles) of product or biomass formed per mass (or moles) of substrate consumed. This is the simplest to calculate practically by measuring the increase in mixed liquor suspended solids (MLSS) and the removal of substrate in the form of biological oxygen demand or chemical oxygen demand (BOD or COD). This is an observed yield as it encompasses all the metabolic reactions and the substrate consumed for cellular processes rather than production of new cells. The yield figure obtained will be distorted slightly due to the presence of inert solids that will be incorporated in to the biomass. The true, stoichiometric or theoretical yield is calculated by stoichiometry and is sometimes considered the maximum possible yield as it represents the yield in the absence of competing reactions (Doran, 1995).

When an organism uses its own organic carbon as a substrate, it is generally defined as endogenous respiration. When considering the activated sludge process, the performance is determined by the activity of the entire biological population, and the endogenous phase of growth for the whole population is described. The effects of endogenous respiration on the net bacterial yield are accounted for by defining the

observed yield which is the net rate of growth (mass per unit volume per time) divided by the substrate utilisation rate (mass per unit volume per time).

Any process capable of reducing the process yield will result in less sludge wasted from the system. Indeed, many process alterations have successfully reduced waste sludge production in the activated sludge process at laboratory scale and will be discussed fully in the following chapters. One area involves the use of chemical inhibitors. Several types of inhibitors exist with the potential to affect activated sludge; uncouplers, tricarboxylic acid cycle inhibitors (TCA) and antibiotics. Metabolic inhibitors have the potential to reduce waste production with the advantage that they are simple to apply. The chemical can be added directly to the aeration chamber so that extra treatment stages are avoided; consequently capital works costs are avoided. The metabolic pathways of any living organism consist of two parts; the catabolic and anabolic. Catabolic pathways are involved with the oxidation of substrate to produce smaller molecular building blocks and energy in the form of adenosine triphosphate (ATP). This molecule is the high chemical energy source for many of metabolic processes including growth and maintenance. The anabolic pathways use the products of the catabolic to allow cells to undergo growth, repair and replication. In tightly coupled cells, (that is operating normally) the synthesis of ATP is associated with substrate oxidation. The ATP synthesis actually regulates the oxidation of substrate, a phenomena termed respiratory control (Hanstein, 1976).

Metabolic inhibitors act in several ways. Uncouplers are a group of simple organic chemicals that destroy the coupling of substrate oxidation to ATP synthesis (Slater, 1963). As a consequence ATP synthesis ceases as it is deprived of the required energy input, and substrate oxidation is uninhibited by respiratory control and continues at maximum rate resulting in heat rather than ATP. Achieving uncoupling in activated sludge microorganisms has the potential to allow breakdown of the pollutants in the wastewater whilst avoiding cell growth and build up of biomass because the anabolic pathways will not have sufficient ATP available (Low and Chase, 1996; Okey and Stensel, 1993).

Antibiotics also interfere with metabolic pathways. Their action is directed towards pathways that are unique to microorganisms and are not found in eukaryotic cells. One significant difference between microorganisms (prokaryotic cells) and eukaryotic (including mammalian cells) is the presence of a cell wall outside the cell membrane in the prokaryotic. Vancomycin inhibits the production of the cell wall mucopeptide and as a secondary effect prevents RNA transcription (Jordan, 1961). Some antibiotics act as uncouplers, others inhibit respiration coupled with phosphorylation such as oligomycin and guanidine (Slater, 1963). The effect of antibiotic addition to sewage treatment and possible subsequent presence in effluent outfalls should be restricted to microorganisms due to their specificity. However, antibiotic resistance and the risk of horizontal transfer to other organisms must be considered which could result in possible harmful environmental effects of the use of such chemicals.

Since the cessation of coastal deposition of sludge in 1998, the current available sludge disposal options commonly used are use on agricultural land, incineration and landfill. Due to the limited outlets and the level of treatment required prior to disposal the costs involved are quite high and expected to rise (Table 1.2).

Table 1.2: Current and predicted costs of main sludge disposal outlets, £/ t dry solids (including processing eg. digestion) Figures supplied by North West Water (Pers. Comm. 1998)

	Use on agricultural land	Incineration	Landfill
1997	136	179	78
2001	228	152	119

With an anticipated 1602 x 10^3 tonnes of sludge in 2000, the disposal could cost the UK in excess of £1900 million. The addition of chemicals into wastewater treatment plants may not be an ideal environmental solution to reducing sludge production in the activated sludge process. The sheer scale of the problem and vast quantities of sludge

to be disposed require a method that results in lowered sludge production: the use of chemical uncouplers has the potential to achieve this and as such is worth investigating. Provided the cost of the chemical required and any other additional costs do not exceed that saved from disposal, chemical uncoupling could prove economically beneficial to the UK water industry.

Successful waste minimisation can have beneficial effects on several aspects of wastewater treatment from environmental concerns through to economical gains (Clark, 1995). The use of chemical inhibitors has the potential to achieve waste biomass minimisation; this project develops their use in the activated sludge process.

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Chapter 2: Synopsis

Chapter 2

Synopsis

Much research has been directed towards developing methods capable of reducing waste sludge production in biological wastewater treatment processes. The success of these methods has generally been reported in terms of the yield coefficient of the process; the lower the yield the lower the amount of waste sludge produced. These methods are explored in detail in Chapter 4. Several techniques were reported to be capable of reducing yield. Systems that promote the growth of specific bacterial predators can significantly reduce biomass output of the activated sludge process. Any system which increases the MLSS of the activated sludge helps to reduce the F:M ratio which will reduce the yield: for example by addition of support matrices to the activated sludge that encourage the adherence and growth of biofilm on their surfaces. Alteration of operating temperature and extended aeration processes also proved successful for reducing biomass accumulation.

Of the techniques investigated for biomass reduction, Chapter 4 reported on the use of chemical inhibitors. This thesis explores the use of such chemicals in activated sludge, since to date, little research has been directed at utilising these chemicals to reduce yield. The potential benefits from using chemical uncouplers stem from the simplicity of the system. Retrofit to existing treatment facilities would not require capital works just a suitable pumping system for chemical addition. Chapter 5 identified several chemicals from literature with the potential to reduce yield in the activated sludge process. For any chemical to be successful in this role the biomass reduction has to be achieved without detriment to process performance. The effect of 12 chemicals on oxygen uptake rate, COD removal and nitrification was determined and described in Chapter 5. Experiments were carried out in an automatic respirometer with 50 ml activated sludge samples. At laboratory scale the oxygen uptake rate was stimulated by chemical uncouplers but reduced by chemicals with an antibiotic action. Generally,

the COD removal rate of chemically treated activated sludge was comparable to untreated controls. The effect on nitrification was varied, some chemicals having negligible effect and others up to 80% inhibition. The kinetic action of the chemicals was briefly addressed and the effect determined to be a mixed type of action with respect to substrate uptake.

The increase in oxygen uptake rate was used as an indicator of the occurrence of uncoupling. The three chemicals that had the greatest effect on oxygen uptake were identified for further investigation. In order to determine the effect of chemical addition on the MLSS, larger scale, fed batch style experiments were carried out. These were operated with a working volume of 600 ml of activated sludge. The sludge was aerated, then settled, the supernatant drawn off and manually fed with settled sewage. Chapter 6 described the effects of trypan blue, rotenone and 2,4 DNP on MLSS; the effect of one single addition of chemical to the activated sludge and regular addition in conjunction with the settled sewage feed was investigated. Reductions of up to 80 % in MLSS were possible with regular additions of rotenone and 2,4 DNP.

Laboratory scale activated sludge simulations were carried out with the most promising chemicals; rotenone and 2,4 DNP. 4 nitrophenol, an uncoupler similar to 2,4 DNP but more powerful was also investigated. The simulations were carried out using 3 l porous pots with a control and test for each chemical studied. In order to maintain a stable operating temperature the porous pots were covered in insulating material and the feed of settled sewage temperature controlled at 20°C. The operating regime was the same for all chemicals under investigation. The MLSS were maintained at 2500 mg l^{-1}. The solids were brushed off the inner walls daily. Samples were regularly monitored to determine the effect of continuous chemical addition on MLSS accumulation, BOD and COD removal. Samples were studied microscopically to determine the effect of chemical addition on the species diversity of the protozoa occurring and the effect on floc size and numbers.

The effect of rotenone in a laboratory scale activated sludge simulation is described in Chapter 7. Despite promising preliminary test results, no significant decrease in activated sludge yield was observed. The chemical presence had no effect on COD or BOD removal compared to controls. The species diversity was not significantly altered.

The addition of 2,4 DNP to activated sludge resulted in a significantly lower yield. This was achieved without detriment to process performance in terms of BOD or COD removal. The full details are discussed in Chapter 8. Although 2,4 DNP is biodegradable, the reduction in yield observed in the course of the experiment suggested that no significant biodegradation occurred.

Addition of 4 NP to activated sludge resulted in a lower yield of biomass compared to the control. The BOD removal was comparable to that of the control, however the COD removal was decreased in the presence of the chemical. Chemical addition resulted in a decrease in the floc size; this may prove detrimental to final effluent quality if the flocs reach 'pinpoint' size and cause decreased settleability in clarification.

From the results of the activated sludge simulations 2,4 DNP was selected as the most appropriate chemical for biomass growth reduction with minimal effect on the activated sludge process performance. Chapter 10 details a pilot scale investigation using 2,4 DNP. Control and test units were identically constructed and operated in the simultaneously. The 330 l aeration vessels were preceded by a 10 l anxoic zone into which the settled sewage feed, return activated sludge and chemical solution were pumped. The activated sludge flowed under gravity into settlement tanks, the return activated sludge was pumped back from the base of the tank and the effluent overflowed from the top. Regular monitoring of COD, BOD, ammonia, nitrate, nitrite, sludge volume index (SVI) and capillary suction time (CST) demonstrated that there was no significant difference between test and control in any parameter. The oxygen uptake rate of the chemically treated activated sludge remained greater than that of the

control throughout the trial. The yield was significantly lower in the chemically treated activated sludge throughout the duration of the trial.

Simple cost analyses carried out in Chapter 11 suggested that despite an increased oxygen demand in 2,4 DNP treated activated sludge and the cost of the chemical, the lower yield reduced the waste sludge treatment and consequent disposal and concurrently the cost. Chapter 11 discussed several mechanisms to ensure that any carry over of chemical into effluent streams may be treated.

Chapter 3: Aims and Objectives

Chapter 3

Aims and Objectives

The aims of this research were

- To identify chemical inhibitors with the potential to reduce biomass production in the activated sludge process.
- To test these chemicals using respirometry to determine their effect on a mixed culture of activated sludge.
- To further investigate potential chemicals to determine the effect on process parameters such as COD removal, MLSS production, nitrification, kinetic action and oxygen uptake rate.
- To identify the most promising inhibitors and to trial in an activated sludge simulation to obtain more detailed results.
- To scale up the trials to a pilot test to determine if the effects seen are sustainable.

Chapter 4: Low Biomass Activated Sludge – a Review

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Chapter 4

Low Biomass Yield Activated Sludge: A Review

4.1 INTRODUCTION

The activated sludge process is widely used throughout the UK and Europe for the treatment of domestic and municipal wastewater. The activated sludge process involves a mixed culture of microorganisms that degrade wastewater aerobically (Bitton, 1994). The suspended biomass is used to treat crude or settled sewage. The microorganisms convert the organic pollutants mainly to carbon dioxide and water, and ammonia to nitrites and nitrates in the presence of oxygen (generally in the form of air). The effluent quality and volume of wastewater were previously of prime importance.

Aerobic wastewater treatment processes are an ideal growth environment for many microorganisms. Due to the variety of compounds present in most wastewaters a diverse ecosystem develops. There are many bacterial species present including *Pseudomonas, Zooglea, Bacillus, Athrobacter, Microthrix, Nocardia, Acinetobacter, Nitrosomonas, Nitrobacter* and *Achromobacter* (Metcalf and Eddy, 1991). Protozoa, rotifers, algae and fungi also contribute to the ecosystem in the activated sludge process. Each of the organisms in such a mixed population have different growth patterns and rates. Such growth patterns depend on various parameters including food, nutrients, pH, temperature and oxygen availability.

4.1.1 Yield

As organisms oxidise organic matter they synthesise new cellular material and undergo growth; substrate utilisation and biomass growth are coupled. The yield of biomass from the activated sludge process is generally stated as a ratio of the biological solids produced, to the mass of the influent organic substrate utilised (often measured as

BOD). These yields are termed observed yields as they are calculated from experimental data and as such encompass the effects of cell decay and maintenance energy. The construction of a mass balance between the use of substrate and biomass growth is vital in the design of biological systems to determine the necessary quantity of material inputs such as nutrients and oxygen and to evaluate the resultant quantity of outputs including waste sludge and carbon dioxide. Very simply, the basic microbial growth equation can be considered as:

$$\text{Carbon source} + \text{energy source} + \text{electron acceptor} + \text{nutrients} \longrightarrow$$
$$\text{Biomass} + \text{carbon dioxide} + \text{reduced acceptor} + \text{end products} \qquad (4.1)$$

Ideally, an equation similar to (4.1), which is in the same form for any situation independent of the type of carbon source, energy source or electron acceptor is required. Use of balanced stoichiometric equations for the overall reaction can achieve a mass balance of the system. The following method originally described by McCarty (1975) incorporates the effects of cell decay and formation of refactory biological residues for suspended growth systems. The method proposed for determining stoichiometric equations for bacterially mediated reactions involved three half-equations (written on an electron equivalent basis). Bacterially mediated reactions consist of a synthesis and an energy component. Consider the following equation for a casein containing waste:

$$C_8H_{12}N_2O_3 + 3O_2 \longrightarrow C_5H_7O_2N + NH_3 + 3CO_2 + H_2O \qquad (4.2)$$

Where $C_8H_{12}N_2O_3$ = casein

$C_5H_7O_2N$ = bacterial cells

Equation (4.2) can be divided into its synthesis and energy components:

$$5/8\ C_8H_{12}N_2O_3 + 1/8H_2O \longrightarrow C_5H_7O_2N + 1/4NH_3 \qquad \text{SYNTHESIS} \qquad (4.3)$$

$$3/8C_8H_{12}N_2O_3 + 3O_2 \longrightarrow 3CO_2 + 3/4\ NH_3 + 9/8H_2O \qquad \text{ENERGY} \qquad (4.4)$$

Such reactions are oxidation-reduction reactions and consequently involve the transfer of electrons. They involve an electron donor (generally the substrate or waste)and an electron acceptor – for each a half reaction can be written:

SYNTHESIS:

$$5/8\ C_8H_{12}N_2O_3 + 65/8H_2O \longrightarrow 5CO_2 + 10/8\ NH_3 + 2OH^+ + 2Oe^- \quad \text{(donor)} \qquad (4.5)$$

$$5CO_2 + NH_3 + 2OH^+ + 2Oe^- \longrightarrow C_5H_7NO_2 + 8H_2O \quad \text{(acceptor)} \qquad (4.6)$$

ENERGY:

$$3/8\ C_8H_{12}N_2O_3 + 39/8H_2O \longrightarrow 3CO_2 + 3/4\ NH_3 + 12H^+ + 12e^- \quad \text{(donor)} \qquad (4.7)$$

$$3O_2 + 12\ H^+ + 12e^- \longrightarrow 6H_2O \text{ (acceptor)} \qquad (4.8)$$

For ease of comparison and use it is simpler to write these half-equations on an electron equivalent basis. This results in the 3 half-reactions below (4.9 – 4.11).

$$\text{Cells } (R_c): 1/20\ C_5H_7O_2N + 2/5H_2O \longrightarrow 1/4CO_2 + 1/20\ NH_3 + H^+ + e^- \qquad (4.9)$$

$$\text{Donor } (R_d): C_8H_{12}O_3N + 13/32H_2O \longrightarrow 1/4CO_2 + 1/16\ NH_3 + H^+ + e^- \qquad (4.10)$$

$$\text{Acceptor } (R_a): \quad 1/2H_2O \longrightarrow 1/4O_2 + H^+ + e^- \qquad (4.11)$$

From these half-equation the overall reaction R can be obtained as follows:

$$R = R_d - f_eR_a - f_sR_c \qquad (4.12)$$

The fraction f_e is the portion of the electron donor which is coupled with the electron acceptor (portion used for energy) and f_s represents the portion of the electron donor which is coupled with cell formation (the portion synthesised). In order for the equation to balance correctly the sum of f_s and f_e has to be equal to 1. In suspended growth reactors f_s and f_e are functions of cell yield and organism decay. The organism decay being a function of sludge age. The cell yield coefficient represents the fraction of the electron donor associated with cell synthesis when sludge age is zero (McCarty, 1975). The equations discussed by McCarty (1975) consider the stoichiometric yield often considered the maximum yield.

Generally in wastewater treatment the organic waste is considered in terms of BOD or COD. Both of these are related to electron equivalent and can be used in half-equation in the same way. The ultimate BOD generally gives the proportion of the COD that is biologically degradable. Thermodynamically by the types of equations discussed above the maximum yield possible is 0.4 kg (dry weight) of cells per kg COD removed. (A simple stoichiometric calculation of the theoretical oxygen demand of propionic acid is set out in Appendix 3).

In this thesis the yields calculated from experimental data are observed yields and include the effects of decay and maintenance in the single equation by monitoring of the MLSS. They are calculated based on equation 4.13. All yields quoted in the following text are in g biomass produced/ g substrate consumed unless otherwise stated.

$$Y = \frac{V \times MLVSS \text{ produced}}{Q \times BOD \text{ utilised}} \qquad (4.13)$$

Where Y = Yield (d^{-1})

V = volume of aeration tank (m^3)

Q = influent flow rate ($m^3\ d^{-1}$)

BOD = biochemical oxygen demand ($kg\ m^{-3}$)

MLVSS = mixed liquor volatile suspended solids ($kg\ m^{-3}$)

For the conventional activated sludge process the yield is about 0.6. (Metcalf and Eddy, 1991). In activated sludge the microorganisms are suspended. Other biological treatment processes involve attachment of the microorganisms to a fixed surface. The trickling filter is one of the most widely used fixed film processes. It is used at 4000 treatment works in Europe and the USA (Gray, 1989). The system has a low energy input as it uses natural ventilation and gravitational flow. A single pass unit has a yield coefficient of 0.3 - 0.5 (Gray, 1989). In biological fluidised beds the microorganisms

are attached to solid particles which are freely suspended in the wastewater. The process has a high energy requirements as pure oxygen is dissolved into the influent to ensure maintenance of aerobic conditions due to high biomass concentrations. The system operates with a yield of about 0.8 (WRc, 1995). Biological aerated filters (BAF) are fixed film processes in which a flooded bed of small media are used for biofilm attachment. Granular media reactors have a yield of 0.63 - 1.06 (WRc, 1995), and structured media reactors a yield of 0.15 - 0.25 (Rutsen, 1993). The activated sludge process is the most widely used biological wastewater treatment for both domestic and industrial plants.

4.1.2 Sludge production

Hopwood and Downing (1965) identified that sludge production was dependent on a large number of factors. They suggested the most important factors were: adsorption or entrainment of biodegradable insoluble matter; biodegradable soluble matter; adsorption of inert suspended and soluble matter; conversion of soluble and adsorbed insoluble degradable matter into bacterial cells; ingestion of bacteria and possibly of other suspended matter by protozoa or other predators; and degradation of microbial cells by endogenous respiration and lysis. Metcalf and Eddy (1991) summarised the important factors affecting the biomass yield of the activated sludge processes being the oxidation state of the carbon source and nutrients; the degree of polymerisation of substrate; metabolic pathways; growth rates and physical parameters.

Presently, member states of the EU operate around 40, 300 wastewater treatment plants that generate 6.5 million tonnes of dry solids a year. The EU sludge production is expected to increase by 50% by the end of 2005, producing 10 million tonnes of sludge a year (Hall, 1996). Up to 65% of the wastewater treatment operating costs are in the preparation for and disposal of waste sludge (Gray, 1989). There are many social, economic and environmental considerations involved in sludge treatment and disposal, which pose a great challenge to the wastewater engineer. With the rising costs and restrictions on sludge disposal the minimisation of biomass yield has become of

increasing importance. Since the nature of the influent wastewater cannot be readily altered, other factors need to be studied to reduce yield.

4.2 BIOMASS YIELD REDUCTION

Waste sludge requires both economical and safe disposal. The problem is increasing as new sewage treatment plants are built and environmental quality standards become more rigorous (Hall, 1996). Treatment of the waste sludge before disposal increases the energy requirements of the wastewater process; reducing the waste output may be economically favourable. Many approaches for reducing biomass accumulation in the activated sludge process have been investigated including, altering process temperature, bacterial predation, addition of support matrices, extended aeration and uncoupling.

4.2.1 Temperature

Ludzack *et al.* (1961) indicated that an increase in temperature from 5 to 30°C increased process efficiency and decreased sludge accumulation. This study only involved investigations at these two specific temperatures. Hunter *et al.* (1966) carried out a similar set of experiments but using a range of temperatures. They aimed to establish how high the temperature could be raised before the process efficiency diminished and the sludge accumulation increased. Activated sludge simulations were fed twice daily with artificial sewage and operated with a retention period of 16 h. Simultaneously the effect of retention time within a range of 4 to 32 h was studied. The effect of temperature on the suspended solids was evaluated by determining the difference between the feed and effluent suspended matter a well as initial, final and wasted MLVSS. A trend towards greater suspended matter destruction with increasing temperature up to a temperature of 45 °C was described. No particular trend was evident in either effluent levels or efficiencies with temperature, though in the range of 20-40 °C BOD removals averaged somewhat higher than suspended matter removal. This study showed that temperature had a significant effect on sludge composition. As the temperature was increased to 45 °C volatile matter and carbohydrate contents

decreased while organic nitrogen content increased. The general effect of increasing the temperature up to 45 °C was better efficiencies and suspended matter destruction with a reversal at 45 °C. This was probably due to adverse effects on the population utilising the synthetic sewage or a trend towards dispersed growth. Hunter *et al.* (1966) also reported that the effect of increasing retention time gave similar trends to that of increasing temperature. At the higher temperatures there was less MLSS accumulation, less filamentous growth and an increased protozoan and rotifer population.

Senez (1962) reported a decrease in both yield and growth rate with increasing temperature up to 37 °C. The respiratory activity of the bacteria *Aerobacter aerogenes* was found to increase until progressive inhibition started above 42 °C. Senez (1962) discussed a critical range of temperatures between which optimal growth can be obtained without total inhibition. In this range, simultaneous decreases of yield and growth rate were found when the catabolic activity was not modified by the temperature. At 40 °C thermal inhibition became evident with a complete inhibition of rate and growth rate observed at 43 °C. Senez (1962) noted yield coefficients of 0.35 at 23 °C and as little as 0.01 at 40.8 °C.

It is not only temperature that affects the observed yield; the rate at which influent particulate substrate and particulate products are generated and the death and decay of microorganisms affects the volatile solids production or the observed yield. Tian *et al.* (1994) used a mathematical model developed by the International Association of Water Pollution Research and Control (Henze *et al.*, 1987) to distinguish between the various particulate fractions of the mixed liquor. To use the model, influent must be characterised into rapidly, slowly and non-degradable portions. Experimental characterisation of the sludge into viable organisms, endogenous decay products and accumulated particulate matter is not feasible. They also measured COD and VSS concentrations during the study. Tian *et al.* (1994) reported that at low temperatures both simulated and experimental results indicated that MLVSS was significantly higher than the corresponding MLVSS at 20 °C regardless of the solids content of the

feed. The actual concentration of viable organisms apparently decreased from 52-63% of the MLVSS at 20 °C to 29-47% at 8 °C. This set of experiments showed that at low temperatures an decreased MLVSS production would occur. In the colder weather the viable organisms will comprise a smaller proportion of the total activated sludge, so require a maintenance of higher MLVSS concentrations (higher retention times) to obtain the same performance as at higher temperatures. Such actions may cause overloading of clarifiers thus higher solids concentrations in the effluent. The viability was determined in terms of the volatile solids present in the mixed liquor. Viability can also be determined by way of plate counting techniques but these may miss dormant, stressed or starved microorganisms that are unable to multiply on conventional laboratory media (Barcina *et al.*, 1995). In this thesis the term viable is used to describe the volatile suspended solids of the MLSS, sometimes known as the active fraction.

4.2.2 Predation of Bacteria

Activated sludge is an ideal habitat for several organisms other than bacteria. One way to reduce excess sludge production would be to maximise and encourage the growth of organisms higher in the food chain which feed on bacteria. This requires the optimisation of conditions for one or more of protozoa, rotifera, oligochaeta and nematoda; all of which prey on bacteria. Macroinvertebrates will use about 90% of assimilated energy over the course of their life cycle (MacFayden, 1963). The encouragement of the growth of these organisms should theoretically minimise the amount of assimilated energy in the treatment processes and reduce overall sludge production. However, in conventional processes growth of free swimming bacteria is suppressed by the presence of the predators themselves *i.e.* those most easily devoured by predators (Gude, 1971). Floc or film growing bacteria, which are selected for in aerobic biological wastewater treatment processes, are more protected from the predators, hence most of the resulting biomass will not be attacked by the predators. To utilise a system like this properly it is necessary to identify predators capable of the

most efficient grazing on the available biomass. Such feeding rates will depend on the size of the organisms concerned (Ratsak *et al.*, 1993).

Investigations into bacterial predation by protozoa and metazoa indicated that considerable yield reduction was possible (Welander and Lee, 1994). Welander and Lee (1994) used two aerobic continuous flow reactors in series, the first favouring free swimming bacterial growth and the second a predation stage. The second stage involved a suspension carrier biofilm of 50 %v/v polyethylene carrier particles. This system gave yields of 0.049. Although successful, the oxygen consumption of the process was almost doubled due to the increase in oxidation of organic matter and increased nutrient discharges occurred in the predation stage.

The probable function of protozoa in wastewater is identified by Gureich and Ladygina (1990) as maintenance of a bacterial population at the condition of physiological youth and in promotion of cell aggregation. In the natural environment nematodes perform a role in enhancement of decomposition rates and stimulation of nutrient regeneration. In sewage decomposition, Abrams and Mitchell (1980) showed the nematode *Pelodera punctata* interacted with the bacteria and caused increased microbial metabolism by grazing. Nematode grazing keeps the bacterial population growing and distributed throughout the sewage substrate so increasing the energy flow and mineralisation of the substrate. The different chemical and physical properties of the nematodes may constitute paths by which substrate is converted to waste. This renders the flocs and filter films more accessible to bacteria so increasing bacterial metabolism and decomposition indirectly (Woombs and Laybourn-Parry, 1986). The effect of nematodes on the decomposition of the sewage substrate both directly and indirectly is related to the numbers of the organism and the feeding rates, respiration and growth for a specific species. The habitat will also affect the influence exerted by the species of nematode. Conversely however, the promotion of predators of microorganisms could result in the over-grazing of selective populations e.g. nitrifiers, which would reduce the diversity of the population and may detrimentally affect process performance. Competition between different bacteria in biological phosphate removal systems has

been reported to be seriously influenced by protozoan and metazoan grazing. This resulted in cessation of the biological phosphorous removal, the main aim of the treatment process (Cech *et al.*, 1994). Such detrimental effects on process performance due to the promotion of predators is not desirable in the activated sludge process.

Woombs and Laybourn-Parry (1986) investigated the contribution and role of bacteriovore nematodes in the energy flow of low rate percolating filter works. By studying feeding rates, energy, ingestion, growth, reproduction and respiration it was determined that energy flow peaked at periods that corresponded to peaks in nematode density and biomass. They reported that the nematode oxygen consumption was only 0.03-2.7 % of the total of the sludge organisms respiration. The direct effect on the process is small, the greatest effect by nematodes is in grazing. Nematodes have the advantage over other predators that the eggs are strongly resistant to classical waste treatment processes such as prolonged aeration, anaerobic digestion and composting (Gaspard *et al.*, 1995).

No single organism is capable of using the wide variety of compounds present in the sewage substrate; this results in a diverse, if artificially created, ecosystem. Use of bacterial predators cause the sludge to be converted to energy, water and carbon dioxide. The biomass is converted from low to high trophic levels; as higher trophic organisms consume the bacteria. During this, the energy of the biomass becomes lower as maintenance and physiological processes use energy leaving less available for biomass production. Predation serves to concentrate the particulate matter in the wastewater and regulate competition between decomposer populations (Ratsak *et al.*, 1994). Using mathematical models, Ratsak *et al.* (1994) investigated the effect of ciliates both at population and individual level proliferate as a function of bacterial biomass. Experimental two stage cascade cultures were used with the second chemostat being a predation trap. Results showed that introduction of the trap gave a decrease of 12-43% in biomass yield compared to a system without ciliate grazing.

Oligochaetes such as *Nais elingus* frequently occur in sewage treatment plants, reproducing asexually by dividing into an anterior and posterior nadid. Biological wastewater treatment requires control of the activity of the various populations present to successfully digest the carbonaceous matter and keep surplus sludge to a minimum. When considering organisms such as oligochaetes, Ratsak *et al.* (1994) discussed the possibility of preventing the organisms from leaving the reactor. This may be achieved by attachment to biofilms or by biomass retention filters. This decreased the food/biomass ratio and consequently leaves less food available for the microorganisms so that sludge production will decline. Ratsak *et al.* (1994) also proposed that stimulation of the higher organisms would help lower sludge production as already stated. Specifically nadids consume large amounts of bacteria. Total organic carbon utilisation ranges from 40-200 mg m^{-3} d^{-2} by *Amphichaeta sannio*, in sewage treatment plants they may form up to 38% of the macroinvertebrates. The use of metazoa to reduce sludge production in trickling filters was successful at a pilot scale where yields were reduced from 0.4 to 0.15 without detriment to nitrification (Rensink and Rulkens, 1997).

Higher organisms or protozoa enhance the reduction of sludge production by introducing an extra grazing stage. This is a further mineralisation in which sludge is converted to new biomass, water and carbon dioxide. Energy losses are incurred due to the inefficient biomass conversion such that optimal conditions achieve maximal loss of energy and minimal biomass production. Ratsak *et al.* (1994) suggested several hypotheses to explain why protozoa enhance the mineralisation of organic compounds, including the C:N:P ratio - excretion of mineral nutrients by protozoa result in accelerated use of carbon sources by bacteria; growth stimulation - excretion of growth stimulating compound by protozoa which enhance the bacterial activity; and grazing effects - a selection of species occurs that can grow fast and inefficiently so increasing the use of carbon sources, along with a decrease of biomass concentration losing energy in the resulting food chain.

4.2.3 Bacterial Parasites and Phages

Along with bacterial predators the interaction of phages and parasites with activated sludge have been studied (Dias and Bhat, 1965). Phages are viruses that use bacterial hosts resulting ultimately in lysis of the host. Bacterial parasites are organisms that feed on the bacteria themselves often resulting in the bacterial cell death. Such organisms have the potential to reduce microorganism numbers by feeding on them and in lytic activity. Specifically, the parasite *Bdellovibrio* and *E. coli* phages were investigated. Phages were found in activated sludge in quantities between 140 and 726 per ml. *Bdellovibrio* against various microorganisms were detected and counted (Table 4.1). Dias and Bhat (1965) used an aerated rotary shaker unit to follow the fate of these parasites in the wastewater treatment process. Neither phage nor *Bdellovibrio* were found to be functional during activated sludge treatment. They reported a ten fold decrease in phage numbers within 2h but they remained constant after this time for approximately 25 h. However this was in contrast to sewage which had large numbers of bacteria other than *Bdellovibrio* that were capable of lysing bacteria.

Table 4.1: Frequency of Parasite Occurrence in Sludge (Dias and Bhat, 1965).

Bacterial species parasitised	No. of parasites found per ml
Pseudomonas aeruginosa	0 - 18
P. fluorescens	86 - 260
Salmonella typhosa	48 - 230
Serratia marcescens	0 - 8
Aerobacter aerogenes	16 - 160

4.2.4 Provision of Support Matrices

Selection of microorganisms that have high maintenance energy could reduce sludge yield. To select such organisms the substrate to biomass ratio should be lowered. This

results in low bacterial growth rate and most substrate is utilised in the maintenance processes leading to low biomass production. The Linpor process lowers the substrate to biomass ratio. It involves the use of highly porous plastic foam particles, which act as a carrier for the active biomass (Morper and Wildmoser, 1990). The particles usually fill about 15-30 % of the aeration volume and follow the hydraulic motions of the water body created by aeration and agitation so are distributed throughout the whole reactor volume. The effect of this support matrix is to increase the MLVSS. Hegemann (1984) ran the Linpor process for 3 years and found no clogging of the carrier material pores by biomass.

4.2.5 Extended Aeration Processes

Sludge reaeration is described as 'the continuous aeration of sludge after its initial aeration in the activated sludge process'. Such processes have been in full scale use since 1917. The more modern designs use approximately 50% of the tank volume for reaeration such as in contact stabilisation or 80% in Ridgewood biological coagulation process (Haseltine, 1961). Haseltine (1961) investigated several processes that have the potential to lower sludge production. They involve the endogenous respiration of cell material and storage products, which will have higher running costs due to increased supply of oxygen. The Hatfield process uses one third the tank volume for reaeration though it may be more efficient to use half of that volume. The return sludge is reaerated before joining the influent sewage. In the Kraus interchange process the return sludge is split with most directly entering the mixed liquor aeration and some to the sludge reaeration tank. Step aeration processes can vary the amount of the tank volume used and are thus quite flexible.

Reddy *et al.* (1983) reported a total oxidation system that allowed a degree of control of the reaction rate in the aeration tank by regulating the recycled sludge. Generally, control of recycle is not practised at extended aeration plants. Often only a baffle separates the settling and aeration chambers. This will account for lower construction costs, but control of the recycle hydraulic flow rate or return sludge is not possible.

Reddy *et al.* (1983) carried out pilot plant studies with one stream with specifically regulated return sludge and the other was allowed to vary naturally. Results showed that control of the return sludge concentration to 10,000 mg l^{-1} in the extended aeration process resulted in successful operation without sludge wastage and with steady output. The system was run for a month and operated without extended hydraulic detention time - a factor normally associated with the process. Yields of nearly zero were obtained as the excess bacteria were lysed, died or were consumed by predators. It should be considered that the studies were only short term and even the authors were unsure if the reactions would continue in an orderly kinetic manner beyond this time. Such pilots showed the possibilities of slow overall growth with excellent treatment whilst providing sludge disposal. This system is essentially two unit processes - activated sludge followed by aerobic digestion - and consequently has very high energy inputs and a high oxygen requirement for the digestion stage.

4.2.6 Uncoupling

Like all living organisms, bacteria have a complex set of metabolic pathways which are vital to life. The catabolic pathways are those in which molecules taken in are reduced in complexity and free energy is made available. These reactions are funnelled into a few common pathways. Microbial catabolism is unique in the diversity of nutrients and mechanisms employed in energy generation (Prescott *et al.*, 1990). Anabolic pathways involve the use of free energy to increase complexity of molecules and build up the structures required by the cell. Many attempts have been made to uncouple the catabolic from the anabolic reaction in the bacteria involved in the activated sludge process. Methods employed include oxic and anoxic cycling, changing the process temperature and the use of chemical addition.

4.2.6.1 Oxic/Anoxic cycling

Chudoba *et al.* (1992) outlined a modified activated sludge system involving an oxic stage, a settling stage and an anaerobic stage in the return sludge line (Oxic-Settling-Anoxic (OSA) system). Their aim was to physiologically stress the microorganisms to

induce uncoupling which is accompanied by a reduction in biomass yield. It was suggested that insertion of the anaerobic phase caused a natural selection of the species of bacteria that were capable of favouring the catabolic pathways. Such bacteria store polyphosphates during aerobic conditions that are later used as energy sources during anaerobic conditions (Chudoba *et al.*, 1992). During the anaerobic phase the bacteria used their intracellular stocks of adenosine triphosphate (ATP) and in 3 h the ATP in the cells was reduced by 40%. Chudoba *et al.* (1992) reported that at higher organic loading in the OSA system, metabolic selection and energetic uncoupling occurred reducing the excess sludge production. When compared to the conventional activated sludge process a reduction of yield from 0.6 to 0.12 was observed. The hydraulic retention time was much longer in the OSA and the system had double the volume. At lower organic loading the specific sludge production remained the same for both OSA and conventional systems.

Adenosine triphosphate plays a key role as an intermediate between substrate oxidation and the biomass synthesis reactions (anabolic). Senez (1962) reported that under some conditions growth rate was limited by the rate of biosynthesis, and that energy produced in excess during microbial metabolism was wasted. This phenomenon was called uncoupled growth. The same pattern was also seen by Stouthamer and Bettenhausen (1977) who concluded that there was a discrepancy between ATP production and its consumption by biosynthesis. In comparison, Harrison and Maitra (1969) and Harrison and Loveless (1971a) showed that uncoupled growth could be induced during the transition between anaerobic and aerobic growth conditions and that yield coefficients were lowered. The transition between anaerobic and aerobic gave yields of 0.3 to 0.4 compared the to 0.43 of the aerobic system alone. Strange *et al.* (1963) reported that anaerobic conditions reduced the intracellular stock of ATP. The transfer from anaerobic to aerobic conditions caused a very rapid synthesis of ATP to compensate. Uncoupling can be achieved by several mechanisms in activated sludge (Table 4.2).

Table 4.2: Energetic uncoupling of catabolism from anabolism resulting in reduced yield

Phenomenon	Method of stress	Reference
Uncoupled growth	Limitation by N source	Senez, 1962
Energetic uncoupling	Anaerobic conditions	Harrison and Loveless, 1971 Strange *et al.*, 1963
Energetic uncoupling	Change in temperature	Senez, 1962 Harrison and Loveless, 1971b
Loss of tight coupling	pH changes	Harrison and Loveless, 1971a
Energetic uncoupling	Pantothenate starvation	Beliach *et al.*, 1972
Energetic uncoupling	Energy dissipation via chemical addition	Low and Chase, 1998

4.2.6.2 Chemical uncoupling

Many chemicals of varying types and mode of action have been reported to cause the metabolic uncoupling of bacterial and eukaryotic cells alike. The common use for uncouplers is in the investigation of metabolic pathways. The antibiotic group of chemicals contains several uncouplers and inhibitors. Many are directed at microorganisms and have medical properties. Cycloserine and bacitracin attack part of the cell wall synthesis mechanism of bacteria (Dawson *et al.*, 1986). This specificity towards bacteria has benefits, as these chemicals will not affect eukaryotic (including mammalian) cells. However, in the medical world the phenomenon of bacterial resistance is well known and wide spread (Harris *et al.*, 1995). Such resistance could occur in the activated sludge system rendering the antibiotic useless for biomass reduction as well as promoting the risk of horizontal transfer of resistance to other organisms. Resistance has built up due to misuse and overuse of antimicrobials, lapses in infection control, increased use of invasive procedures and devices and widespread use in agriculture and animal husbandry (File, 1999). Equally, there are examples in agriculture where antibiotics have been used in animal feeds since the

1950's for developing intensive farming successfully without documentation of resistance (Thomke and Elwinger, 1998).

Beliach *et al.* (1972) investigated the effect of pantothenate on the bacterial species *Zymomonas mobilis.* Starvation of pantothenate acted as limiting factor and resulted in a decrease in growth rate. This was followed by a decrease of the molar growth yield of glucose. Beliach *et al.* (1972) reported that in all cases the rate of catabolic activity was constant. This implies that the effect of the chemical was an uncoupling of the anabolic and catabolic paths.

As well as unique bacterial pathways, chemical addition can affect more universal metabolic systems. Rotenone, 2,4-Dinitrophenol (2,4 DNP), antimycin A, dicoumarol and oligomycin all uncouple oxidative phosphorylation (Dawson *et al.*, 1986) disrupting the specific energetic link between catabolism and anabolism. Alloxan and cyanide interfere with the TCA cycle. Some of these chemicals such as cyanide are too toxic to man to investigate their use in the activated sludge system. Several of the others have the potential to cause uncoupling of metabolism and thus a reduction in biomass yield.

2,4 DNP over time has been used a dietary aid, an insecticide, weed killer, a dye and a wood preservative. Although toxic in high concentrations, many studies have reported that at concentrations of around 1 to 25 mgl^{-1} it is a powerful stimulator of respiration. This stimulation was accompanied by an inhibition of growth. Rich and Yates (1955) investigated the effect of 2,4 DNP on activated sludge on a laboratory scale. It was found that initial removal of organic matter by the activated sludge was stimulated by the addition of 2,4 DNP and that the stimulation increased with increasing concentration of chemical. After 2 h Rich and Yates (1955) reported that a concentration of 25 mgl^{-1} slowed activity of the sludge to a point where the removal of organic matter by the control was equal to or greater than the test sample. The experimental work also showed that the settling of the sludge and washing with synthetic sewage 'lost' the effect of the 2,4 DNP. The effects on the permanency and

concentration lead to the possibility of further use of this inhibitor. However. no attempt in this study was made to look at the biomass yield with the addition of the chemical. Low and Chase (1996) investigated 2,4 DNP in a single strain of *Pseudomonas putida* and determined it to be less effective than para-nitrophenol. Using para-nitrophenol Low and Chase (1996) achieved inhibition of biomass production without suppression of substrate uptake. The control samples gave yields of 0.32 to 0.35 and treated with 30 mgl^{-1} para-nitrophenol a yield of 0.15.

Investigations with 2,4-dichlorophenol (2,4-DCP) which has the same basic skeleton as 2,4 DNP showed, at bench scale, an increase in respiration and reduced substrate uptake by a factor of 2 to 5 (Okey and Stensel, 1993). Uncoupling occurred but with detriment to the substrate uptake. Okey and Stensel (1993) also noted two accounts of uncoupling at full scale works. The effect of the unidentified uncouplers was an increase in oxygen requirement, a modest decrease in the substrate uptake and a net biomass synthesis of essentially zero. These few studies have shown that chemical uncoupling was possible in both specific bacteria and in mixed populations such as activated sludge and has the potential to reduce biomass.

4.3 DISCUSSION

A reliable and realistic method is needed for biomass reduction in the activated sludge. The average yield for the activated sludge process is around 0.6 (Metcalf and Eddy, 1991) and the yield coefficients obtained from the different methods of process manipulation range from 0 to 0.5 (Table 4.3).

The biological systems of the microorganisms are important in the activated sludge process. Most biological processes are temperature sensitive and it is suggested that one method of increasing process efficiency would be to operate at higher temperatures. Domestic wastewater volumes and climatic temperatures may mitigate against such a process change. Increasing process temperature was effective at biomass reduction (Senez, 1962). However the increase to 47 °C required may not be feasible outside the laboratory nor economical. The range described by Senez (1962)

was very precise, control to these specific temperatures required for optimising the decrease in yield would be difficult in the changing climate conditions faced. To achieve such temperatures at a full scale works would require a massively increased energy input.

Table 4.3: Yield coefficients of biomass reduction processes

Method of biomass reduction	Yield coefficient	Reference
Increasing temperature	0.35 at 23 °C	Senez, 1962
	0.01 at 47 °C	Senez, 1962
Bacterial predation		
- by protozoa and metazoa	0.049	Welander and Lee, 1990
- by ciliates	0.07 - 0.23	Ratsak *et al.*, 1994
- by metazoa	0.15	Rensink and Rulkens, 1997
Extended aeration	0	Reddy *et al.*, 1983
Oxic/anoxic cycling	0.5	Malnou *et al.*, 1984
	0.25	Chudoba *et al.*, 1992
	0.3 – 0.4	Harrison and Maitra, 1969
Chemical uncoupling	0.15	Low and Chase, 1996
Ozonation	0	Yasui and Shibata, 1994

As a natural system the predation of bacteria by both protozoa and metazoa has been widely investigated. Although considerable yield reduction is possible (Welander and Lee, 1994) conditions have to be controlled to promote the growth of the most appropriate predator according to the activated sludge population present. Generally a second aerated chamber is required to promote the predator growth (Ratsak *et al.*, 1994) which, compared to the conventional activated sludge process will involve higher operating costs in the form of oxygen requirements and possibly capital works to install such a chamber. Due to the increased nutrient outputs during the predation stage, further processing may be necessary to meet discharge consents depending on

the extent of the nutrient output, another additional cost. Along with predation, bacterial parasites and phages have the potential to decrease biomass accumulation. These are naturally occurring in activated sludge (Dias and Bhat, 1965) but little work has been directed at their exploitation for biomass reduction. A recent review investigated strategies for biomass reduction in wastewater treatment that encourage further metabolism of organic carbon to respiration products rather than biomass. This encompassed methods such as grazing and lysis, however no recent research into the use of phages or parasites was reported suggesting that such systems were not successful for this application (Low and Chase, 1999).

Manipulation of the microorganisms already in the activated sludge could prove a simple way of reducing biomass yield. Selection of those species with a high maintenance energy, that is use most of the energy derived from wastewater substrate breakdown for cellular maintenance and endogenous metabolism, could reduce the biomass produced. This selection can be achieved by lowering the substrate to biomass ratio such as by the provision of support matrices in the Linpor process. The Linpor process has been employed to improve process efficiency with respect to BOD removal and higher organic loadings but no attention was paid to the effect on biomass yield (Morper and Wildmoser, 1990).

Yields of essentially zero are possible by extended aeration (Haseltine, 1961; Reddy *et al.*, 1983). Initial construction costs can be avoided by merely separating the aeration and settling chambers with a baffle however, running costs are considerable due to the major energy demand in the form of oxygen. It is in effect two unit processes and consequently has very high energy inputs (Reddy *et al.*, 1983). Efficient in terms of biomass production, the running costs have to be compared to the sludge disposal costs to determine the economical basis of this process.

The uncoupling of microbial metabolism has been achieved by the transition of the microorganisms from anaerobic to aerobic growth (Chudoba *et al.*, 1992; Harrison and Maitra, 1969; Harrison and Loveless, 1971a & b). Systems including oxic, settling and

anoxic stages are commonly used in biological phosphorus removal on an industrial scale (Malnou *et al.*, 1984; Fukase *et al.*, 1985; Cooper *et al.*, 1994). Yields of 0.5 are reported from such phosphorus removal plants. When specifically set up to reduce biomass oxic/anoxic cycling can reduce yields to 0.25 (Chudoba *et al.*, 1992). As sewage treatment plants of the appropriate design and operation are already in existence, with optimisation for both nutrient removal and prevention of biomass accumulation oxic/anoxic cycling may be a suitable solution for biomass reduction.

Metabolic uncoupling by chemical addition into the activated sludge negates the need for alterations to the treatment plant. Present work both on single cultures (Low and Chase, 1996) and at bench scale on a mixed culture (Okey and Stensel, 1993) indicates considerable yield reduction with little effect on process efficiency. Optimisation and correct dosing are important to avoid microbial cell kill instead of uncoupling and so careful plant management would be necessary (Okey and Stensel, 1993; Low and Chase, 1996). The increase in oxygen demand found will cause an increase in operating costs (Okey and Stensel, 1993).

In the normal running of the activated sludge process the substrate is converted by oxidation to carbon dioxide water and new biomass. Very simply, considering glucose as a substrate, using the formula for biomass to be $C_5H_7O_2N$ and the yield to be 0.6;

$$C_6H_{12}O_6 + 1.2\ O_2 + 0.96\ NH_3 \longrightarrow 0.96\ C_5H_7O_2N + 1.2\ CO_2 + 4.8\ H_2O \quad (4.14)$$

Ideally, chemical uncoupling could achieve zero yield that is the substrate is completely oxidised to carbon dioxide and water and no new biomass is produced:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \quad (4.15)$$

From these basic stiochiometric equations the increase in oxygen demand is apparent, five times greater for the total oxidation process. In any process with zero yield such an increase is likely.

Chemical traces in the effluent may be toxic or interfere with the effluent consents to be met. Final processing, such as ozonation may be required in order to 'clean' the effluent. In plants where processes like ozonation are already in use, chemical uncoupling may be the simplest form of biomass reduction as it requires no other plant or process changes.

All the methods discussed have drawbacks and the solution may lie elsewhere. Genetic engineering of the microorganisms has the potential to reduce biomass yield and enhance xenobiotic degradation (Singleton, 1994). Two genetically engineered *Pseudomonas sp* were introduced into activated sludge microcosms with the same level of aeration, nutrient availability and microbial community structure as activated sludge reactors (Nusslein *et al.*, 1992). These microorganisms had modified catabolic paths designed to degrade specific substrates (3 chlorobenzoate, 4 methyl benzoate and toluene) not normally broken down by microorganisms. The genetically engineered microorganisms (GEMs) survived in the environment and achieved degradation of the chemicals and were able to pass on the modified genetic material to other organisms in the community. Modification of the metabolic routes to prevent new growth may also be able to avoid the increased energy demands and operating costs. Presently, the deliberate release of genetically engineered microorganisms is environmentally unacceptable and raises many questions about uncontrolled spreading of genetically engineered material (Hoekstra, 1990). In order for GEMs to reduce biomass production in the activated sludge process as many species in the mixed culture have to be altered as possible, which may not be logistically or economically viable on a full scale.

The need to reduce biomass production in the activated sludge process continues to grow as the turnover of treatment plants increases and legislation for disposal tightens. The potential to manipulate the process to reduce yield is increasing and each individual methodology has its advantages. The most efficient and cost effective solution has yet to be optimised.

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Chapter 5: The Effect of Metabolic Inhibitors on Activated Sludge

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Chapter 5

The Effect of Metabolic Inhibitors on Activated Sludge

5.1 INTRODUCTION

The activated sludge process is in widespread use for the treatment of municipal wastewater; the main objective of which is the aerobic removal of carbonaceous matter via suspended growth of heterotrophic microorganisms. As the system is aerobic, investigating the oxygen uptake rate of the mixed liquor can monitor success of pollutant biodegradation and microbial activity (Therien *et al.*, 1984). Respirometric techniques can be employed to assess the toxicity of a waste stream to a microbial population by observing oxygen uptake rate (OUR). The set up of respirometers varies, though all are based on some technique for measuring the rate at which biomass takes up dissolved oxygen (Spanjers *et al.*, 1998). Respirometers can use an open system or a closed system. Open respirometers are generally aerated for a period of time so that the microorganisms reach the endogenous phase (when dissolved oxygen reaches a baseline level). The aeration is then ceased and the effect of a pulse of substrate or toxicant addition investigated by monitoring the dissolved oxygen concentration. Closed cell respirometers are often coupled to a manometer and sometimes an electrolysis cell (such as the system used in this research). Closed cell systems continuously monitor the oxygen consumption of a population of microorganisms and are operated such that neither the aeration rate nor the concentration of dissolved oxygen can limit the biological activity (Therien *et al.*, 1984).

Respirometers can be implemented on line in a treatment works or used as a separate laboratory tool. Respirometry can be used as a screening technique to determine the effect of chemical addition on the function of the mixed population of activated sludge (Ros, 1993). Recent applications of respirometry in wastewater treatment include the development of Respiration Inhibition Kinetics Analysis to determine and quantify the

effect of xenobiotics on carbon removal (Volksay and Grady, 1990). Further development of this type of procedure has resulted in the RODTOX biosensor, which can determine the effect of toxicant addition on carbon and nitrogen substrates (Kong *et al.*, 1996). This system consisted of an open, automated respirometer with a 10 l aerated activated sludge vessel for offline assessment of inhibition kinetics.

Microorganisms function using a complex set of metabolic pathways (Prescott *et al.*, 1990). Interaction with these pathways by physically or chemically stressing the bacteria introduces methods to control the growth and reproduction of the microorganisms. The system by which microorganisms derive chemical energy from substrate and build it into the energy rich phosphate bonds of adenosine triphosphate (ATP) is via a set of oxidation/reduction reactions (oxidative phosphorylation). Electrons move along a transport chain in which oxygen acts as the final acceptor in aerobic respiration. In tightly coupled cells, very little oxidation occurs in the absence of ATP synthesis. Uncouplers are able to remove the coupling of substrate oxidation and ATP synthesis resulting in ATP synthesis halting and substrate oxidation being uninhibited by respiratory control (Hanstein, 1976).

Investigations using the chemical uncoupler para-Nitrophenol (4 NP) on a pure culture of *Pseudomonas* suggested uncoupling was an efficient method of reducing biomass yield (Low and Chase 1996). The use of uncouplers in activated sludge has the potential to increase process efficiency in terms of waste sludge production with few plant alterations and running costs via control of the individual microorganisms involved. These chemicals are only suitable if biomass reduction can be achieved without detriment to the pollutant oxidation rates and efficiencies of the process. This study investigated the effect of various chemical uncouplers and inhibitors on oxygen uptake rate to identify occurrence of uncoupling. Observation of the effect on general process performance parameters including oxygen uptake rate, removal of organics, ammonia removal and nitrate production was studied.

5.2 MATERIALS AND METHODS

All experiments were carried out using activated sludge from Cotton Valley sewage treatment works (Anglian Water Plc) and maintained with domestic settled sewage from Cranfield University sewage treatment works. Predetermined concentrations of chemicals were added to 50 ml samples of mixed liquor and oxygen uptake analysed with an electrolytic respirometer (Model 017, CES Ltd Kent) at 20 °C, data being logged every 5 min. The CES Ltd. Aerobic respirometer No. 017 uses an electrolytically coupled manometer system. It is a closed system (that is, no external influences once the cell is set up) which measures pressure changes in the gaseous exchange of the sample. A carbon dioxide trap (of a concentrated hydroxide solution) absorbs the carbon dioxide given off by the respiring cells and as such there is a pressure drop measured in the manometer. This causes the electrolyte to rise in the manometric electrolysis cell and touch the sensor stimulating electrolysis. Once the production of oxygen has restored the pressure in the vessel the electrolysis ceases. The molar ratio of the carbon dioxide produced to the oxygen consumed is used to calculate the amount of oxygen utilised by the sample in mg against time which is automatically logged by the respirometer (Gillard, 1996).

The chemicals tested were in three groups: uncouplers affecting the ATP transfer specifically 2,4 dinitrophenol (2,4 DNP) (97%), 4 NP (98%), rotenone (97%), dicoumarol (99%), quinacrine dihydrochloride hydrate (98%), chlorpromazine hydrochloride (98%) (figures in brackets indicate purity of chemical supplied); antibiotics with bacterial specific action (vancomycin hydrochloride hydrate, erythromycin hydrate, oligomycin (mix of A, B, C 90%), antimycin A) and tricarboxylic acid cycle (TCA) inhibitors (congo red, trypan blue). All chemicals were supplied by Sigma Aldrich (Dorset, UK). Information from literature was used to calculate approximate chemical doses that would induce uncoupling without cell kill (Table 5.1). For example as vancomycin gives cell kill at 5 - 40 mgl^{-1}, a range of concentrations of 1 – 20 mg l^{-1} were used to obtain biomass reduction without total cell kill. The chemicals were made into solution with water so that no other chemical

addition was made to the activated sludge and any effect could be attributed directly to the chemical inhibitor. Solubility varied from chemical to chemical (Table 5.2).

Table 5.1: Available literature data for chemical inhibitors

Chemical	Literature data	Source
2,4 DNP	5 mg l^{-1} for 1300 - 1500 mg l^{-1} MLSS is sufficient for uncoupling	1
Quinacrine	uncouples at 10^{-3} M	2
Rotenone	50 % inhibition at 10 *p* mol mg^{-1} protein	2
Vancomycin	achieves *S. aureus* kill at peak concentrations of 20 -40 mgl^{-1} and trough concentrations of 5 – 10 mgl^{-1}	2
Congo red	toxic at molar concentration of 1.2×10^{-8}	2
Trypan blue	1 in 16000 gives 92 % inhibition	2
Chlorpromazine	inhibits at 10^{-3} M	2
Dicoumarol	uncouples at 2×10^{-5} M	2
Erythromycin	50 % inhibition at 0.04 μg l^{-1}	2
Antimycin	50 % ihibition at 0.5 μM	3
Oligomycin	50 % inhibition at 1 μg mg^{-1} protein	3
4 NP	100 mg l^{-1} reduced yield to 0.2 without detriment to uptake of substrate	4

Sources: 1. Okey, R and Stensel, D. 1993
2. Dawson *et al.*, 1986
3. Franklin, T. J and Snow, G. A 1989
4. Low E. and Chase H. 1996

Initial trials aimed to achieve suggested concentrations for approximately 50 - 100 % uncoupling. Doses of chemicals were calculated as final concentration in mg l^{-1}; if data was available for a mass to cell mass ratio this was used according to MLSS, and then final concentrations calculated (Dawson *et al.*, 1986; Verscheuren 1996). To investigate the effect of inhibitor addition on COD removal, glucose solution was added to 50 ml activated sludge to make 0.5 g l^{-1} solution and the samples aerated for 2

h. Mixed liquor suspended solids and COD were measured according to standard methods (APHA, 1992). The effect of various concentrations of inhibitor on the nitrification ability of the activated sludge was determined using a standard method (HMSO, 1980).

Table 5.2: Solubility of chemcial inhibitors

Chemical	Solubility
2,4 DNP	Soluble in water/100 mg ml^{-1} in ethanol
Quinacrine	50 mg ml^{-1} in water
Rotenone	0.2 mg ml^{-1} in water
Vancomycin	50 mg ml^{-1} in water
Congo red	1 mg ml^{-1} in water
Trypan blue	1 mg ml^{-1} in water
Chlorpromazine	50 mg ml^{-1} in water
Dicoumarol	5 mg ml^{-1} in water
Erythromycin	Sparingly in water / 50 mg ml^{-1} in ethanol
Oligomycin	Partially in water / 50 mg ml^{-1} in acetone
4 NP	Soluble in water / 25 mg ml^{-1} in methanol

The kinetics of most enzymatic reactions can be represented by Michaelis–Menten kinetics. These look at the initial velocity (V_0) of a reaction of substrate to product catalysed by a given concentration of enzyme under constant reaction conditions; and initial velocity varies with the concentration of supplied substrate ([S]) (Doran, 1995). The relationship between V_0 and [S] is definable in terms of an equation for a rectangular hyperbola:

$$V_0 = \frac{a\,[S]}{[S] + b} \qquad (5.1)$$

Where a and b are constants.

Experimental application leads to the constants a and b being defined as V_{max} and K_m respectively. This form of the equation is the Michaelis- Menten equation:

$$V_0 = \frac{V_{max}[S]}{[S] + K_m} \qquad (5.2)$$

V_{max} is the maximum rate or velocity of reaction at infinite reactant concentration and K_m is the Michaelis constant for reactant (or substrate) S, which is only described in experimental terms and is equal to the value of [S] at which $V_0 = ½ V_{max}$. As this is difficult to determine experimentally, a linerisation of the Michaelis-Menten equation is used (the Lineweaver – Burke linerisation):

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \times \frac{1}{[S]} \qquad (5.3)$$

However, this linearisation distorts the experimental error in V, which is amplified further at low substrate concentration and consequently often gives inaccurate results and is not ideal for determining V_{max} and K_m (Doran, 1995). As the experiments to be carried out here involved low substrate concentrations the least amount of distortion in the errors inherent in the biological system was desirable. Of the other linearisations available, the Langmuir Plot gives the minimum distortion in experimental error (Doran, 1995). It is obtained by multiplying the Lineweaver – Burke equation by S:

$$\frac{V}{[S]} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \qquad (5.4)$$

If a reaction obeys Michaelis-Menten kinetics a plot of [S]/V against [S] gives a straight line with a slope of $1/V_{max}$ and intercept of K_m/V_{max}.

Consequently, Michaelis-Menten kinetics were investigated using fixed chemical concentrations and increasing amounts of substrate. The velocity of reaction (V) was

measured as oxygen consumption rate (mg mg^{-1} min^{-1}) and substrate concentration [S] was the concentration of glucose added per ml (mg ml^{-1}). Langmuir plots of S/V against S were plotted and used to calculate the K_i and V_{max} from the axis intercept and slope respectively. The units of K_i were mg ml^{-1} and those of V_{max} being min^{-1}; as it is the mass of substrate consumed per unit mass of enzyme present per time unit.

5.3 RESULTS

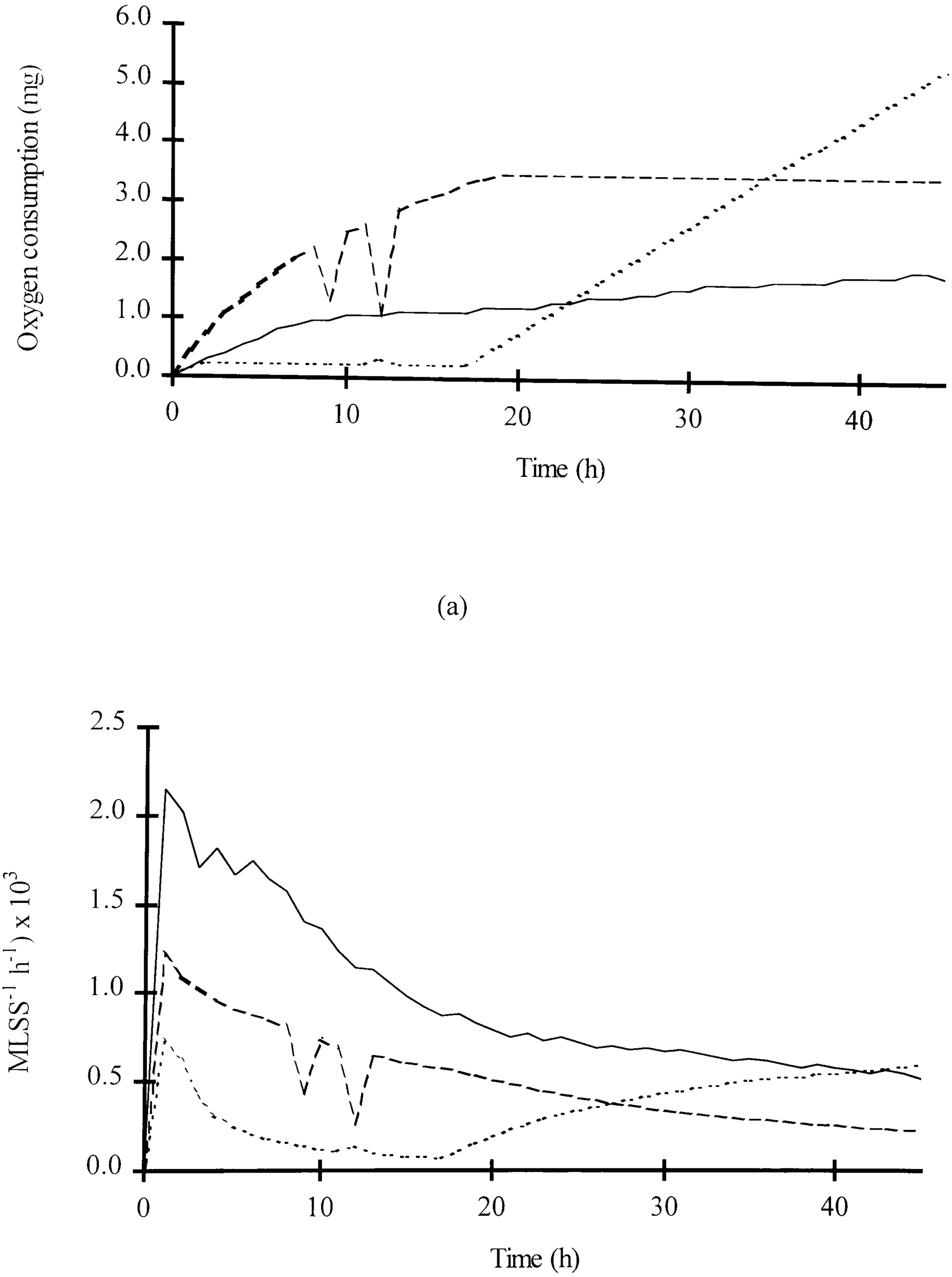
The effect of chemical addition on oxygen uptake rate varied according to the class of chemical i.e. antibiotic, uncoupler or TCA inhibitor. Oligomycin showed an increase in the amount of oxygen consumed compared to the control (Figure 5.1 (a)). However, the rate of utilisation was less than that of the untreated activated sludge (Figure 5.1 (b)). Vancomycin also reduced the rate of oxygen uptake compared to the control (Figure 5.1). Addition of chemical uncouplers to activated sludge caused an increase in both the amount of oxygen utilised and the rate of consumption compared to the control (Figure 5.2). The increase suggested that uncoupling was successful; increased substrate breakdown involving further oxygen consumption occurred in order to try to meet the energy demands of the anabolic pathways which had been halted by the addition of the uncouplers. Results showed that rotenone increased the rate more than the addition of 2,4 DNP when both were compared to the control (Figure 5.2).

The four antibiotics all caused a decrease in the oxygen uptake rate compared to their respective controls (Table 5.3, Figure 5.1 (b)). Control samples were run with each test since the activated sludge was collected at different times, and as such had different microbial populations, MLSS concentration and consequently different respiratory activity. Presence of the antibiotics will reduce the number of cells respiring and reduce the rate of uptake. Antimycin reduced the uptake from 2.1 mg O_2 g $MLSS^{-1}$ h^{-1} in the control to only 0.1 mg O_2 g $MLSS^{-1}$ h^{-1}, suggesting that cell kill may be occurring instead of inhibition. Initially, vancomycin had a very low oxygen consumption and rate (Figure 5.1) but around 17 h after treatment both utilisation and rate increased compared to than the control. Since no additional substrate was added

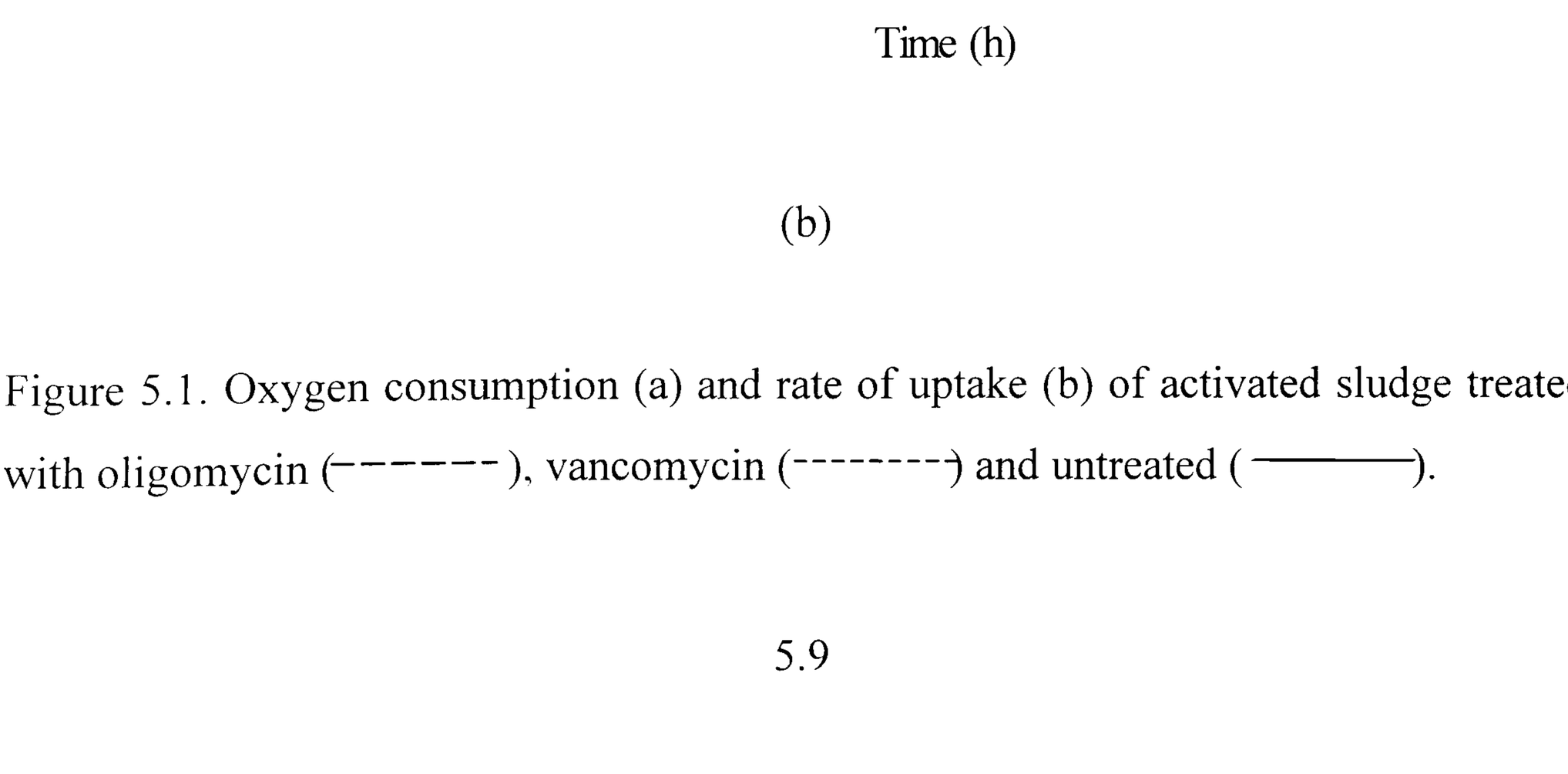
after the start of the tests it was possible that uncoupling of the endogenous metabolism had occurred.

Of the two TCA inhibitors, congo red halved the oxygen uptake compared to the control whereas trypan blue caused a small increase in rate. The three more powerful uncouplers (4 NP, 2,4 DNP and rotenone) increased oxygen consumption compared to the control. The increase in oxygen utilisation suggested that uncoupling had successfully been achieved. If catabolism is uncoupled from anabolism, further substrate is broken down to produce ATP to meet the energy demand of the anabolic pathway. This demand was not met due to the inhibitor action and increased oxygen uptake was seen as substrate oxidation was promoted to yield more ATP.

After 1 h exposure, rotenone and 2,4 DNP gave the greatest oxygen uptake rates (17.0 and 13.5 mg O_2 g $MLSS^{-1}$ h^{-1} respectively) which dropped to 6.5 mg O_2 g $MLSS^{-1}$ h^{-1} and 3.0 mg O_2 g $MLSS^{-1}$ h^{-1} after 24 h; both still greater than the controls. Over this 24 h time period no further substrate was supplied and the increased rate seen early on will have exhausted the substrate, the fall in rate occurring as depleting substrate became the limiting factor. 4- nitrophenol caused a small increase in the rate of uptake but after 34 h showed a decrease compared to the control.

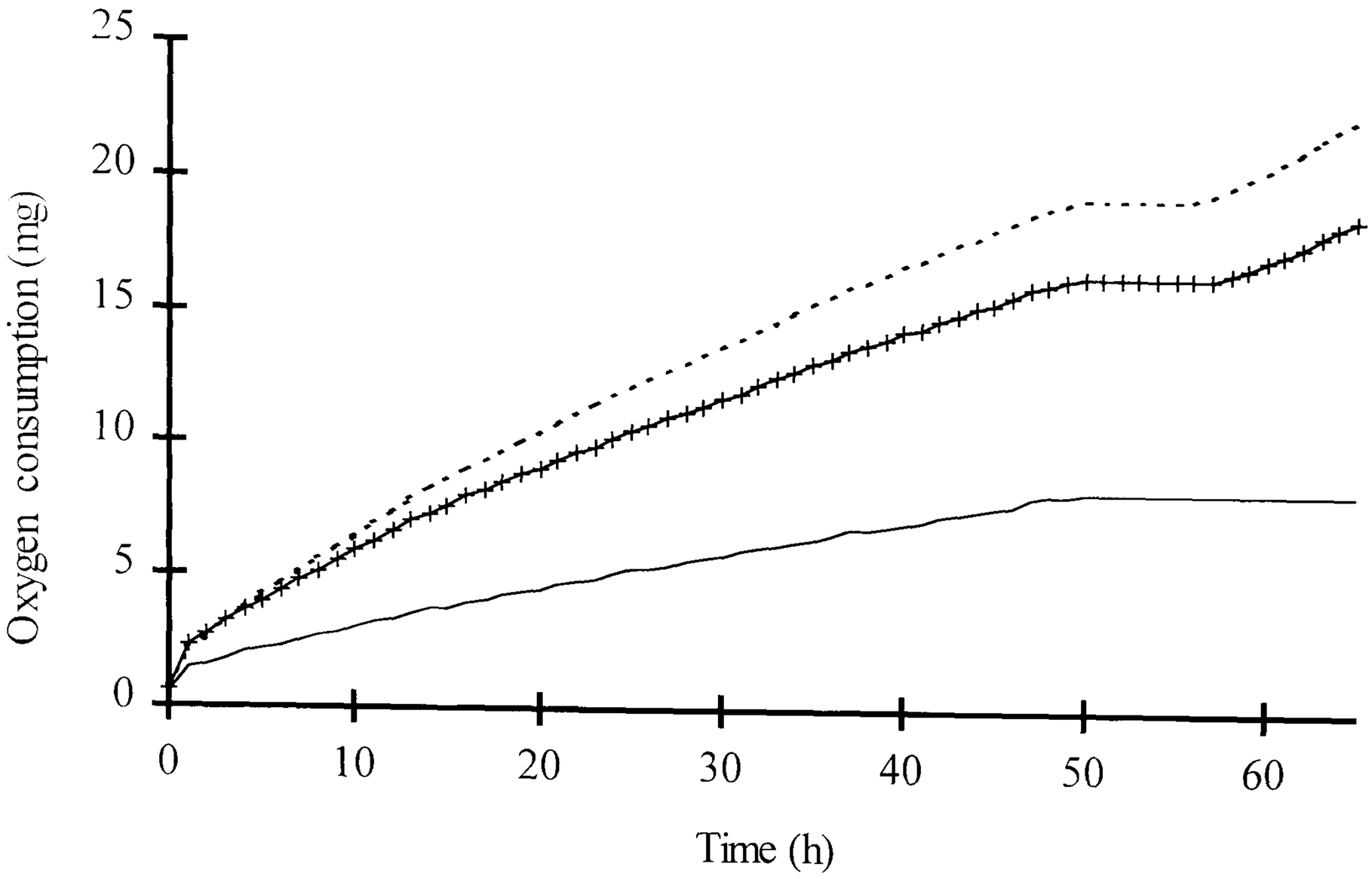


(a)

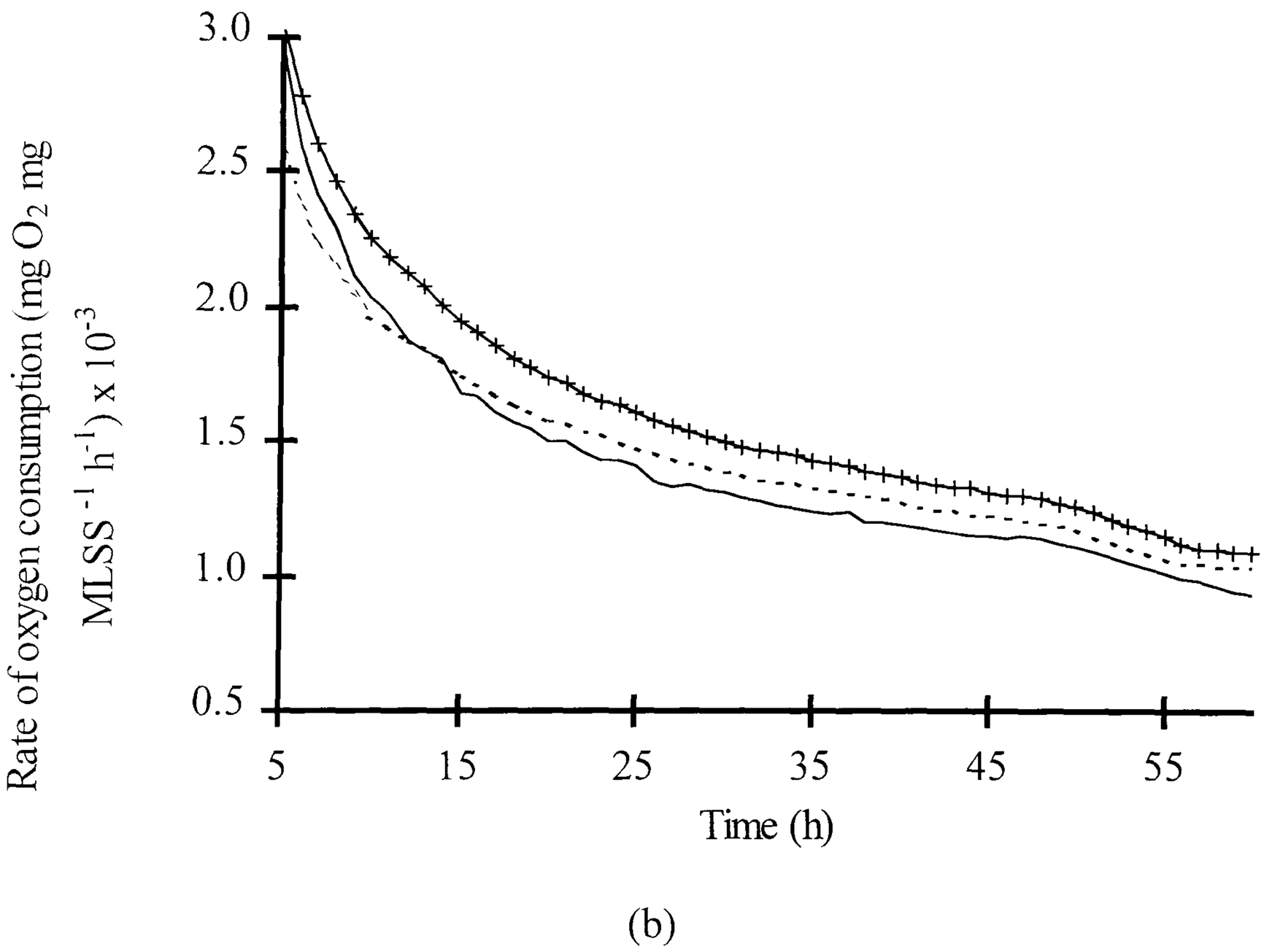


(b)

Figure 5.1. Oxygen consumption (a) and rate of uptake (b) of activated sludge treated with oligomycin (– – – – – –), vancomycin (- - - - - - - -) and untreated (————).



(a)



(b)

Figure 5.2: Oxygen consumption (a) and rate of uptake (b) of activated sludge treated with 2,4 DNP (+), rotenone (.....) and untreated (———).

The other chemicals with an uncoupling action (dicoumarol, chlorpromazine and quinacrine), resulted in only an insignificant increase in uptake rate or even a small reduction suggesting that at the concentrations used, uncoupling was insufficient or unsuccessful. The chemicals showed varying effects on the oxygen uptake rates which may have been dependent on the chemical mode of action, the number of microbial species inhibited in the culture and the effectiveness of the chemical at inhibition or uncoupling.

Table 5.3: Rate of oxygen uptake (mg O_2 g $MLSS^{-1}$ h^{-1}) of activated sludge treated with chemical inhibitors (summary)

		CHEMICAL		CONTROL	
Chemical	Dose mgl^{-1}	Time exposed 1 h	Time exposed 24 h	Time exposed 1 h	Time exposed 24 h
2,4 DNP	4.0	13.5	3.0	9.4	2.8
4 NP	30.0	4.9	0.7	3.7	1.8
rotenone	4.0	17.0	6.5	9.4	2.8
dicoumarol	4.0	3.6	2.2	3.5	2.8
quinacrine	800.0	1.1	0.3	4.2	0.5
chlorpromazine	1000.0	2.1	1.1	1.4	5.2
vancomycin	1.0	0.7	0.3	4.2	0.5
erythromycin	0.04	3.3	3.4	2.1	5.2
oligomycin	2.0	2.4	0.4	2.1	0.4
antimycin	2.74	0.1	1.4	1.4	0.4
congo red	0.01	0.7	0.2	1.7	0.5
trypan blue	18.0	2.9	0.7	1.4	0.4

The mean COD removal of untreated activated sludge was 0.02 ± 0.01 kg COD kg $MLSS^{-1}$ h^{-1}, over a 2 hour experimental duration. The uncouplers rotenone and 2.4 DNP both appeared to improve COD removal, rotenone resulting in 0.42 ± 0.26 kg

COD kg MLSS $^{-1}$ h^{-1} with a dose of 6.0 mg l^{-1}, 20 times greater than the control. The only chemical to actually increase the COD concentration was dicoumarol (Table 5.4). The standard deviations about the mean reported (Tables 5.4 and 5.5) were very large as a result of experimental procedure. The activated sludge was collected fresh from a local works for each run of tests and as such different in composition for each repeat of chemical treatment. Treatment of the activated sludge with antibiotics or TCA cycle inhibitors caused little or no reduction in the COD removal capabilities. At higher concentrations vancomycin appeared to enhance the COD removal (0.032 ± 0.016 at 2.5 mg l^{-1} addition) (Table 5.5). In general, the addition of chemical inhibitors to activated sludge did not reduce the ability of the system to remove COD over the short term.

Table 5.4: Mean COD removal rate of activated sludge treated with chemical uncouplers, n = 4 ± S.D. (kg COD kg MLSS $^{-1}$ h^{-1})

2,4 DNP		4 NP		Rotenone		Dicoumarol	
Dose mg l^{-1}	Rate kg COD kg MLSS $^{-1}$ h^{-1}	Dose mg l^{-1}	Rate kg COD kg MLSS $^{-1}$ h^{-1}	Dose mg l^{-1}	Rate kg COD kg MLSS $^{-1}$ h^{-1}	Dose mg l^{-1}	Rate kg COD kg MLSS $^{-1}$ h^{-1}
2.0	0.106 ± 0.106	10	0.018 ±0.024	2.0	0.018 ±0.024	4.0	-0.044± 0.363
4.0	0.079 ± 0.085	30	0.015 ± 0.029	4.0	0.275 ± 0.226	6.8	-0.005± 0.246
6.0	0.068 ± 0.064	50	0.018 ± 0.012	4.5	0.082 ± 0.047	8.0	0.057± 0.130
8.0	0.088 ± 0.099	80	0.006 ± 0.024	6.0	0.419 ± 0.267	9.6	0.137± 0.100

Table 5.5: Mean COD removal rate of activated sludge treated with TCA inhibitors and antibiotics n = 4 ± S.D. (kg COD kg MLSS $^{-1}$ h^{-1})

Trypan blue		Vancomycin		Erythromycin	
Dose	Rate	Dose	Rate	Dose	Rate
mg l^{-1}	kg COD kg MLSS $^{-1}$ h^{-1}	mg l^{-1}	kg COD kg MLSS $^{-1}$ h^{-1}	mg l^{-1}	kg COD kg MLSS $^{-1}$ h^{-1}
10	0.024 ± 0.014	0.5	0.008 ± 0.002	0.05	0.022 ± 0.007
20	0.020 ± 0.012	1.0	0.017 ± 0.002	0.07	0.018 ± 0.018
30	0.023 ± 0.01	2.5	0.032 ± 0.016	0.10	0.018 ± 0.016
40	0.014 ± 0.008	5.0	0.022 ±0.003	0.12	0.013 ± 0.026

Concurrent with carbonaceous matter removal the activated sludge process removes ammonia via nitrification. The mean rate of ammonia removal by the control (untreated) activated sludge was 2.4 ± 1.1 mg NH_4 g $MLSS^{-1}$ h^{-1}, and the mean rate of nitrate production was 1.3 ± 0.6 mg NO_3 g $MLSS^{-1}$ h^{-1}. At all the concentrations tested, all the chemicals caused a mean reduction in the rate of ammonia removal (Figure 5.3). Rotenone and trypan blue both caused an increase in the rate of nitrate production (Figure 5.3), the other chemicals (2,4 DNP, 4 NP, erythromycin and vancomycin) all reduced the rate of nitrate production.

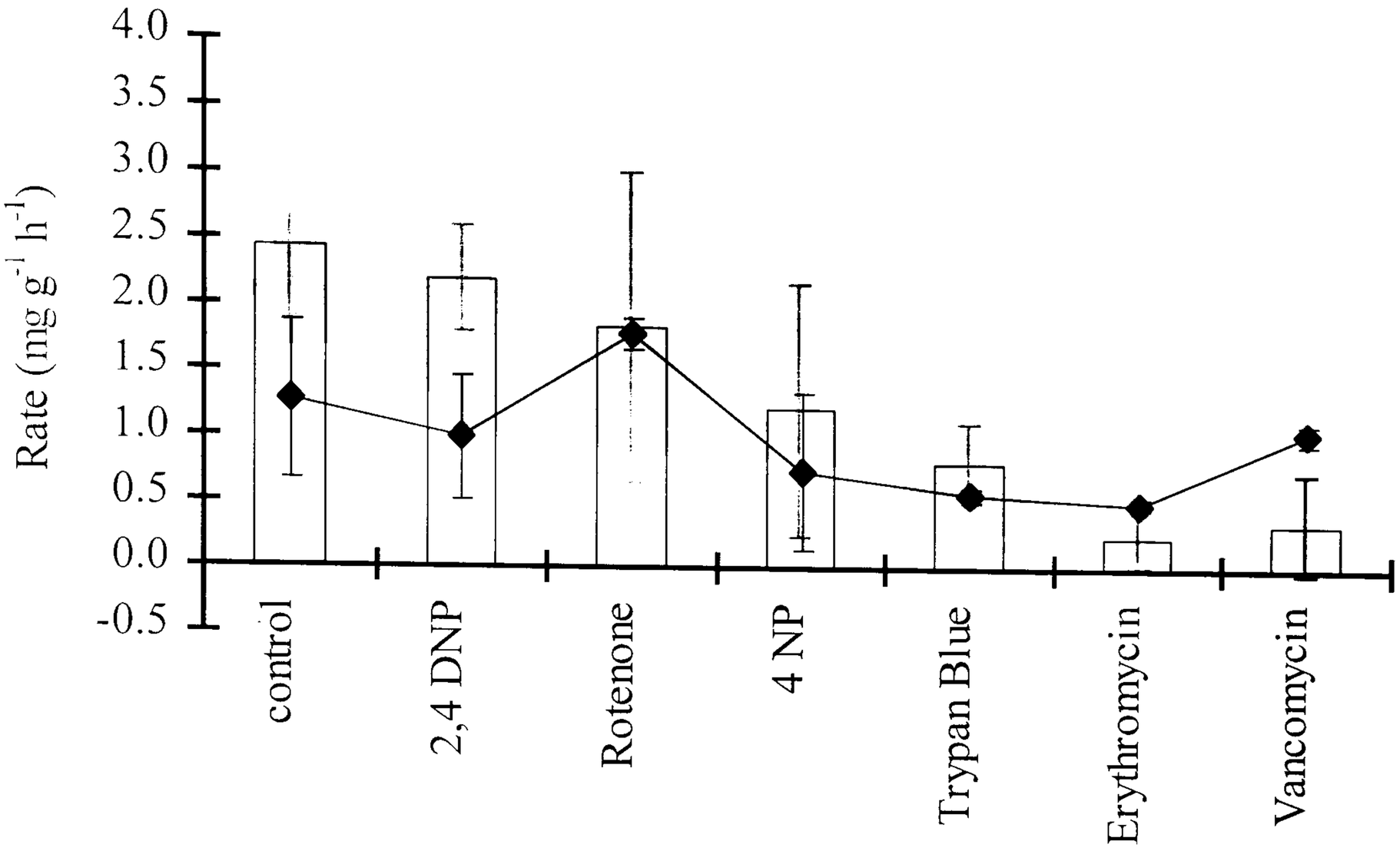


Figure 5.3: Mean rate of ammonia removal (bars) and nitrate production (line) of chemically treated activated sludge (including standard deviation). Means are of all concentrations of chemcial tested.

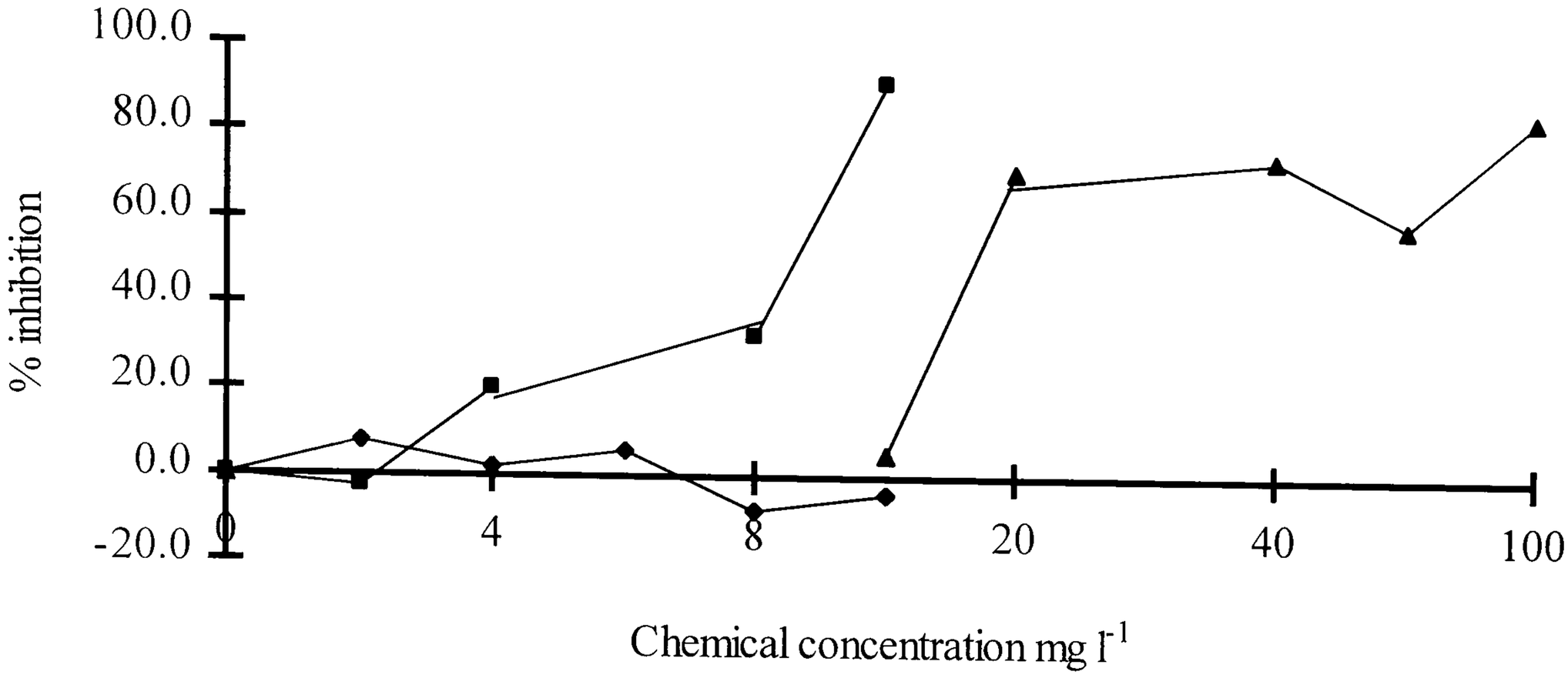
Each chemical was tested at several different concentrations. The reduction in ammonia removal or nitrate production did not increase with increasing chemical concentration but remained relatively constant. In terms of actual nitrification occurring as measured by ammonia removed and nitrate produced over the course of the experiment increasing chemical concentrations further increased the inhibition of nitrification.

Trypan blue (a TCA inhibitor) caused significant reduction in the nitrification ability of activated sludge reducing ammonia removal by upto 60 % and nitrate production by 30 % (Table 5.6). The antibiotics vancomycin and erythromycin both inhibited nitrification; erythromycin by 100 %, even at low concentrations (Table 5.6).

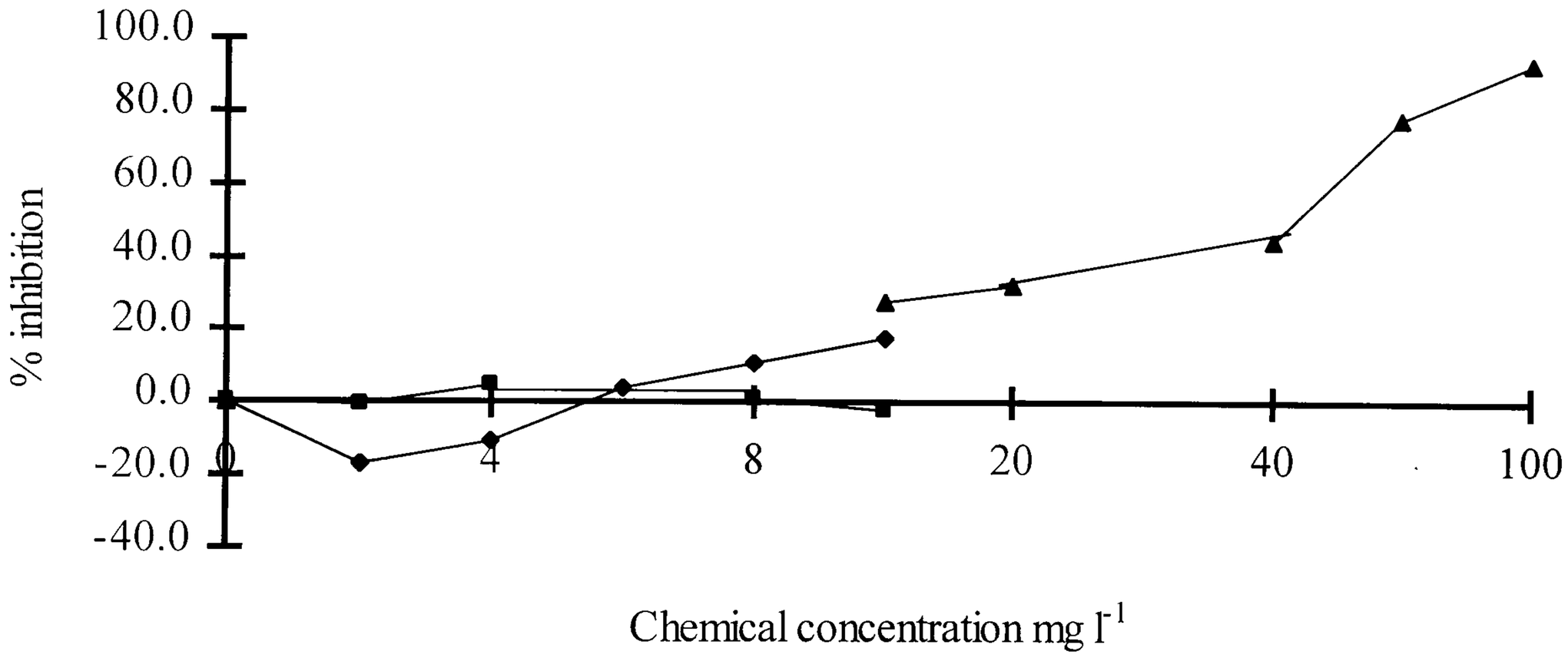
Table 5.6: Inhibition of nitrification (%) in activated sludge treated with chemical inhibitors compared to control (in terms of both ammonia removed and nitrate produced)

Erythromycin			Vancomycin			Trypan blue		
	% inhibition			% inhibition			% inhibition	
Dose mgl^{-1}	NH_3	NO_3	Dose mgl^{-1}	NH_3	NO_3	Dose mgl^{-1}	NH_3	NO_3
0.05	100	-1.7	1.0	9.5	46.3	10.0	45.8	-3.3
0.10	64.4	13.3	5.0	16.0	38.9	20.0	30.5	26.7
0.12	69.5	10.0	7.0	13.5	53.7	30.0	62.7	30.0

Increasing concentrations of 4 NP caused increasing inhibition of nitrification in terms of both ammonia removal and nitrate production by up to 80% (Figure 5.4). Rotenone, which resulted in the greatest uncoupling, also exhibited increasing inhibition of ammonia removal with concentration up to 90%. However, rotenone had little effect on the amount of nitrate produced; less than 10 % inhibition. 2,4 dinitrophenol had the least impact on nitrification of all the chemicals tested causing only 8 % reduction of ammonia removal even at higher chemical concentrations. At the concentration identified for uncoupling, a negligible inhibition of 1.6 % occurred. The presence of 2,4 DNP appeared to stimulate the production of nitrate at lower concentrations.



(a)



(b)

Figure 5.4: % inhibition of nitrification of activated sludge treated with chemical inhibitors (a) by ammonia removal and (b) by nitrate production (◆) 2,4 DNP (▲) 4 NP (■) rotenone (negative numbers suggest a promotion rather than inhibition)

Michaelis-Menten kinetics were applied to look at the effect of metabolic inhibitor addition to glucose substrate removal. Linearisation of the Michaelis-Menten equation in the form of Langmuir plots of S/V against S (Figure 5.5) were used to calculate K_i

and V_{max}. Inhibitor presence caused an alteration in both the slope and the intercept in Langmuir plots.

Addition of any chemical at any dose caused a decrease in the apparent V_{max} as calculated from the Langmuir plots. Trypan blue resulted in the largest reduction of V_{max}(Table 5.7). Increasing concentration of 2,4 DNP from 4 to 6 mg l^{-1} reduced the V_{max} but a further increase in chemical concentration to 8 mg l^{-1} did not result in further reduction. Quinacrine, chlorpromazine, trypan blue and vancomycin all resulted in lower K_i values than the control. Treatment with 4 mg l^{-1} 2,4 DNP caused an increase in K_i, but increasing 2,4 DNP concentration resulted in lower values than the control.

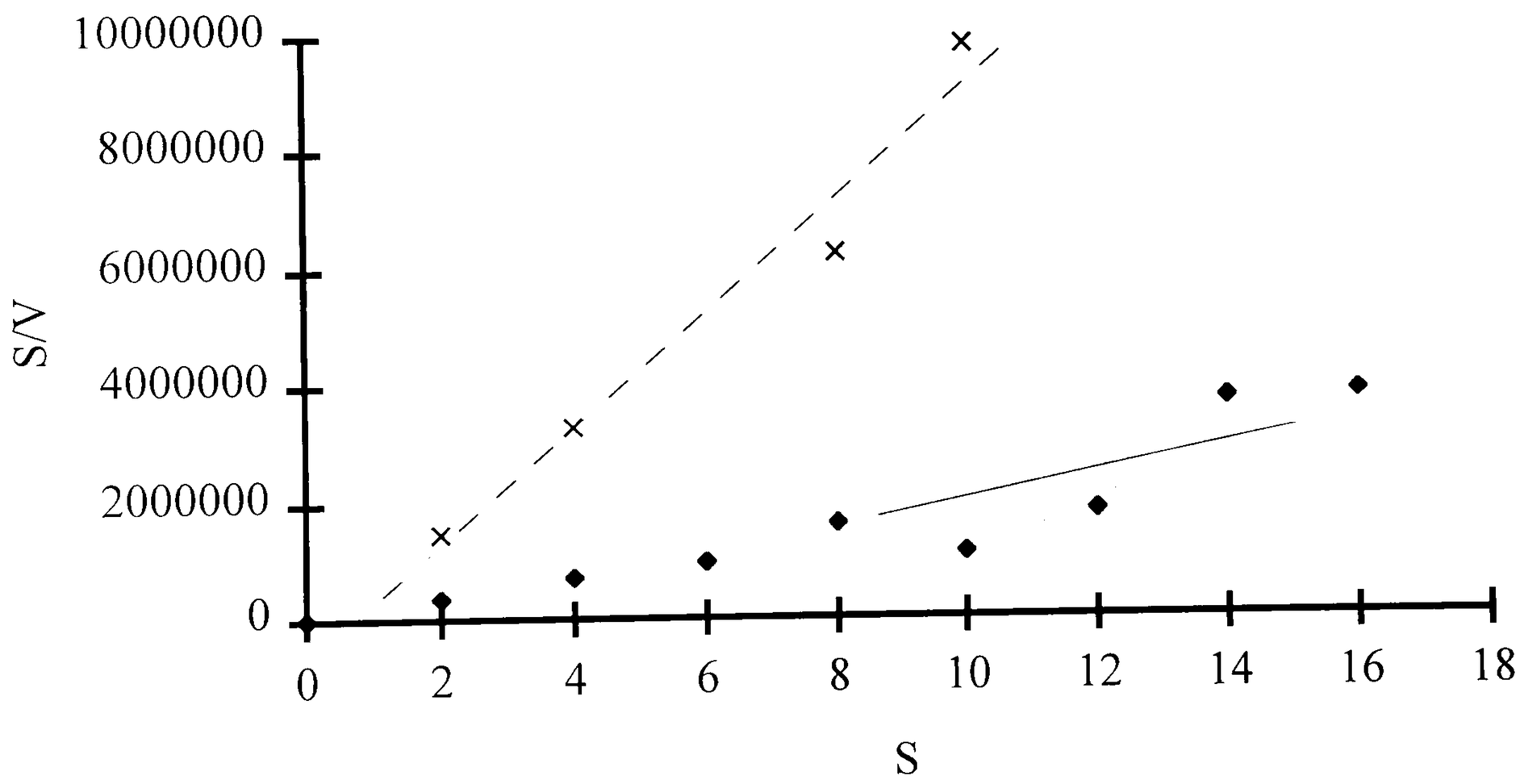


Figure 5.5: Langmuir linerization of Michaelis-Menten kinetics: control (◆) and 8 mgl^{-1} (✖) 2,4 DNP treated activated sludge

Table 5.7: K_i and V_{max} values of activated sludge treated with various chemical inhibitors

Chemical	Dose mg l^{-1}	K_i mg ml^{-1}	V_{max} min^{-1}
Chromium III[1]	100.0	0.1	-
4 nitrophenol[2]	10.0	0.5	-
control	-	1.2	5.5×10^{-6}
2,4 DNP	4.0	1.8	1.0×10^{-6}
	6.0	0.7	4.6×10^{-7}
	8.0	0.5	1.9×10^{-7}
Rotenone	5.0	3.5	1.1×10^{-6}
Quinacrine	400.0	1.0	1.6×10^{-7}
	500.0	-1.5	2.0×10^{-7}
Chlorpromazine	320.0	0.5	1.3×10^{-6}
	500.0	-1.8	8.4×10^{-7}
Trypan blue	20.0	1.5	9.4×10^{-7}
Vancomycin	1.0	-2.5	2.2×10^{-6}

[1]Saida and Abdelkader, 1994

[2] Haghighi-Podeh and Bhattacharya, 1996

5.4 DISCUSSION

Previous research has identified that the addition of chemical uncouplers to cell suspensions has the potential to reduce biomass accumulation (Low and Chase, 1997; Okey and Stensel, 1993). Theoretically, there is great potential for application in wastewater treatment but it has yet to be fully explored. These experiments were designed to rapidly screen many chemicals to determine which, if any were suitable for biomass reduction in the activated sludge process. Several important process parameters involved in wastewater treatment were investigated at a laboratory scale: oxygen uptake, COD removal and nitrification.

Simple respirometric tests observed the effect on oxygen uptake. An increase in oxygen uptake suggested that uncoupling was occurring and this implied a concurrent biomass reduction. The uncouplers (2,4 DNP, 4 NP, rotenone and dicoumarol) all increased the oxygen uptake rate of activated sludge compared to control samples. Of these, rotenone and 2,4 DNP caused the greatest increases suggesting the greatest extent of uncoupling and hence biomass reduction. Both rotenone and 2,4 DNP are uncouplers disrupting the transfer of energy from catabolic to anabolic paths in the form of ATP (Dawson *et al.,* 1986). It is the production of the ATP that controls cell respiration rate. Addition of uncouplers removes this control, respiration rate increases until intracellular substrate reserves are used. The rate of oxygen uptake is influenced by both the stimulating effect of the uncoupler and the metabolism of exogenous substrate. Toxicity at high concentrations was not observed with any of the chemicals tested as the doses were calculated to achieve inhibition without cell death to allow other cellular processes to occur and sewage treatment continue. Increased endogenous respiration was observed with cultures developed from settled sewage and soil inoculums grown on phenol treated with both di and mono chlorophenols (Okey and Stensel, 1993).

Literature suggested that groups of chemicals other than uncouplers had the capability of reducing biomass accumulation. These other chemicals were antibiotics and TCA cycle inhibitors. The antibiotics caused a decrease in the rate of oxygen uptake compared to controls. Antibiotics have specific action towards bacteria or a bacterial pathway, which is not always the ATP link between catabolism and anabolism. The reduction in the respiration rate was expected as it was probably due to the antibiotic action reducing the numbers of cells respiring. Vancomycin has been observed to inhibit the formation of a cell wall mucopeptide in *Staphylococcus aureus* (Jordan, 1961) by preventing cell wall repair or new cell formation. Oligomycin inhibits a membrane bound form of ATP-ase preventing the phosphoryl group transfer (Franklin and Snow, 1989) acting much like an uncoupler interfering with energy production via ATP.

Reduction in the rate of oxygen uptake suggested that wastewater treatment will be detrimentally affected. If less oxygen was consumed it is likely that less carbonaceous matter can be aerobically broken down. The efficiency of the treatment process for organic pollutant matter breakdown is generally measured in terms of BOD or COD removed. Historically, BOD has been measured but this is a time consuming analysis and in order to obtain a more rapid insight into the effect of these chemicals on organic removal COD removal was investigated. Activated sludge process response to uncouplers in terms of substrate uptake (COD removal) varied widely according to the uncoupler and concentration applied. Rotenone and 2,4 DNP increased the rate of COD removal by upto 20 times. Antibiotics and the TCA cycle inhibitors had little or no effect on the rate of removal. The only chemical to increase the COD of the sample was dicoumarol. Substrate depletion studies using 2,4 dichlorophenol (Okey and Stensel, 1993) showed a considerable decrease in the zero order uptake rate of phenol and glucose; a reduction substrate uptake rate was observed for all the chemicals investigated: 0 - 20% of phenol was degraded over a 300 min time period compared to the control utilising 100 %. Conversely, oxidation of organic matter by activated sludge treated with 2,4 DNP was stimulated with increasing chemical concentration (Rich and Yates, 1955). Addition of the uncoupler 4 NP has been reported to stimulate the average specific rate of substrate degradation whilst successfully lowering yield (Low and Chase, 1997). Uncoupling or inhibition of growth was successful in these experiments without a negative effect on substrate removal.

Nitrification, the first stage in the process by which nitrogen is removed biologically from wastewater involves two bacterial genera; *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* oxidises ammonia to an intermediate product nitrite, which is then converted to nitrate by *Nitrobacter* (Metcalf and Eddy, 1991). Often, subsequent tertiary treatment stages are used to achieve nitrification, however, increasingly activated sludge systems are operated to achieve nitrification in conjunction with carbonaceous removal. The rate of ammonia removal was reduced in the presence of the chemicals tested, most chemicals also caused a reduction in the rate of nitrate production. The greatest percentage inhibition of nitrification occurred with the

treatment of 4 NP with up to 90% according to chemical concentration. Nitrate and nitrite are metabolites of 2,4 DNP when degraded by several organism species (Lenke *et al.*, 1992; Verschueren, 1996). If the 2,4 DNP added was being utilised as a secondary substrate an increase in the nitrate production would be expected. At low concentrations, a negative percentage inhibition occurred and it was possible that the microorganisms utilised the chemical as an alternative carbon source, releasing nitrate as breakdown product, thus increasing the nitrate concentration. However, overall 2,4 DNP caused negligible inhibition of nitrification; the only chemical under test to have had such a minor effect on ammonia removal.

Careful chemical and dose selection can result in stimulation of oxygen uptake rate and biomass reduction, an increase in COD removal and have no significant effect on nitrification.

A wide range of chemicals were screened all giving variable results in terms of potential for biomass reduction in the activated sludge process. The use of Michaelis-Menten kinetics to investigate the two rate constants K_m and V_{max} was aimed at trying to identify any patterns between the chemical action. If all the successful inhibitors follow the same inhibition pattern then identification of other chemicals as yet untested may be possible. For reversible inhibitions in enzyme kinetics, there are 3 classifications; competitive, non-competitive and uncompetitive. Competitive inhibition involves the adsorption of the inhibitor at the substrate binding sites and thus direct competition with the enzyme for substrate (Bailey and Ollis, 1986). Non competitive inhibitors do not affect the enzyme-substrate binding affinity but form complexes that can not break down to yield products. Uncompetitive inhibitors combine with forms of enzyme that do not themselves combine with substrate. The addition of chemicals caused the alteration of both the slope and intercept of the plots suggesting that the action is mixed (competitive and non-competitive) (Bailey and Ollis, 1986). All the chemicals tested reduced the constant V_{max}. The parameter used to determine V_{max} was oxygen uptake rate. Since addition of the chemicals caused an increase in oxygen uptake rate it may have been expected that V_{max} would increase

also. The effect on K_i was varied resulting in a range of values from –2.5 to 3.5. Research with chromium (III) in activated sludge suggested that for doses of 100 mg l^{-1} a K_i of 0.11 mg ml^{-1} occurred which was a similar magnitude to those values found in this study (Saida and Abdelkader, 1994).

Okey and Stensel (1993) stated that the response of activated sludge to an unknown uncoupler was an increase in endogenous respiration rate, reduced synthesis when metabolising growth substrate, reduction in the rate of usable substrate uptake and toxicity at high concentrations. This study confirmed that several uncouplers increased the rate of oxygen uptake and resulted in reduced synthesis but without a reduction in the rate of usable substrate (in terms of COD). Therefore, 2,4 DNP showed the greatest promise for lowering biomass yield in the activated sludge process. Uncoupling i.e. degradation of wastewater without cell growth and without cell death, in mixed cultures of activated sludge was successfully achieved with 2,4 DNP. This was indicated by the increased oxygen uptake, concurrently COD removal and nitrification were not detrimentally affected.

5.5 CONCLUSIONS

- Several chemicals including uncouplers, TCA cycle inhibitors and antibiotics had potential for reducing biomass accumulation in activated sludge.
- Uncouplers increased the oxygen uptake rate of activated sludge whereas antibiotics resulted in a reduction, both actions suggested successful reduction in biomass.
- Little detrimental effect on the COD removal capability of the activated sludge was found with chemical addition, except dicoumarol, which increased the COD concentration. Stimulation of the rate of removal occurred with rotenone and 2,4 DNP.
- The effect of chemical addition on nitrification was varied. Least inhibition of ammonia removal and nitrate production occurred with 2,4 DNP.
- Enzyme kinetics suggested that the chemical action was of mixed inhibition on the activated sludge.

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Chapter 6: Chemical Uncoupling – The Effect on Batch-Fed Activated Sludge Units

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Chapter 6

Chemical Uncoupling - The Effect on Batch-Fed Activated Sludge Units

6.1 INTRODUCTION

Several chemicals were identified that could interact with the mixed culture of activated sludge with limited affects on COD removal and nitrification ability (Chapter 5). The key parameter of a chemical inhibitor in activated sludge is its ability to reduce biomass production. 2,4 dinitrophenol has been previously reported to reduce solids accumulation in the activated sludge process (Rich and Yates, 1955). 2,4 dinitrophenol is an uncoupler and exhibited an increase in oxygen uptake rate (Chapter 5), the only other uncoupler to promote such an increase in oxygen uptake rate was rotenone. Trypan blue acts on a specific metabolic pathway (TCA cycle) and addition of this to activated sludge increased oxygen uptake rate. As these chemicals appeared to achieve the greatest extent of uncoupling according to oxygen uptake rate reported in the previous chapter, the effect of these chemicals on the MLSS was investigated.

All previous testing had been at a very small scale and bench tests were needed to assess the effects of the metabolic uncouplers on a larger scale. Laboratory scale sequencing batch reactors were used as they were simple both in design and operation. In these experiments, simple vessels were used which were fed sewage once daily rather than a continuous flow (termed here as batch-fed) and were manually operated as sequencing batch style reactors. There are 5 phases involved in sequencing batch reactors: fill, react, settle, draw and idle. These can be operated automatically or as in these simple experiments manually. The operational flexibility allows an ability to meet many different treatment objectives and such reactors are often used on much larger scales (Ketchum, 1997).

6.2 MATERIALS AND METHODS

Additional respirometry tests were carried out on activated sludge (Cotton Valley sewage treatment works, Anglian Water Ltd) to confirm that the chemical concentrations selected were capable of uncoupling without cell kill. The 50 ml sample was dosed with inhibitor, stabilised and held at 20 °C, stirred continuously and oxygen uptake measured automatically every 5 min (Model 017, CES Ltd, Kent, UK). The chemicals were supplied by Sigma Aldrich Ltd (Dorset, UK). The chemicals were applied with final concentrations of: rotenone 4.0 mg l^{-1}, 2,4 DNP 4.0 mg l^{-1}, dicoumarol 0.8 mg l^{-1} and trypan blue 18.0 mg l^{-1}. Further respirometry testing investigated the effect of the chemical on ability to use substrate (glucose). 50 ml activated sludge samples were dosed with inhibitor and glucose solution (0.1 g ml^{-1}) (Merck, UK) added to obtain 230 mg l^{-1} COD, the samples were held at 20 °C and monitored in an automatic respirometer for oxygen uptake.

To determine the effect of the chemical addition on larger samples, activated sludge was maintained in a batch-fed operation. Activated sludge samples of 600 ml were dosed with one treatment of trypan blue, 2,4 DNP or rotenone at final concentrations of 18, 4 and 4 mg l^{-1} respectively (or 0.01, 0.67 and 0.57 mg g $MLSS^{-1}$ respectively) plus one untreated control. The activated sludge was aerated and mixed continuously. Daily, all the sludge samples were settled and 300ml supernatant removed and replaced with settled sewage. A replicate set of treated samples for both chemicals were set up at the same time. On the daily addition of the settled sewage, an extra dose of the chemical was added according to the volume of fluid removed to maintain the final concentration (resulting in a total dose over 11 days of 33.0 mg g $MLSS^{-1}$ trypan blue, 0.96 mg g $MLSS^{-1}$ rotenone and 1.4 mg g $MLSS^{-1}$ 2,4 DNP). Influent and effluent (removed supernatant) samples were monitored daily for COD. MLSS of the activated sludge was carried out daily. The MLSS of the samples were determined according to standard methods (APHA, 1992).

6.3 RESULTS

Respirometry of the activated sludge indicated that the chemical presence caused an increase in the amount of oxygen utilised by the sample (Figure 6.1). The amount of oxygen utilised increased compared to the control depending on the chemical and dose (Figure 6.1). Of those tested, 2,4 DNP and dicoumarol increased the oxygen uptake to the greatest extent. This indicated successful uncoupling of catabolism and anabolism. All chemicals caused at least a doubling in the amount of oxygen consumed at 20 h. The rate of oxygen uptake was greater for the chemically treated activated sludges (Table 6.1). Rotenone and 2,4 DNP exhibited the greatest rates after 2 h, however at 12 and 20 h all chemical treatments had similar oxygen uptake rates.

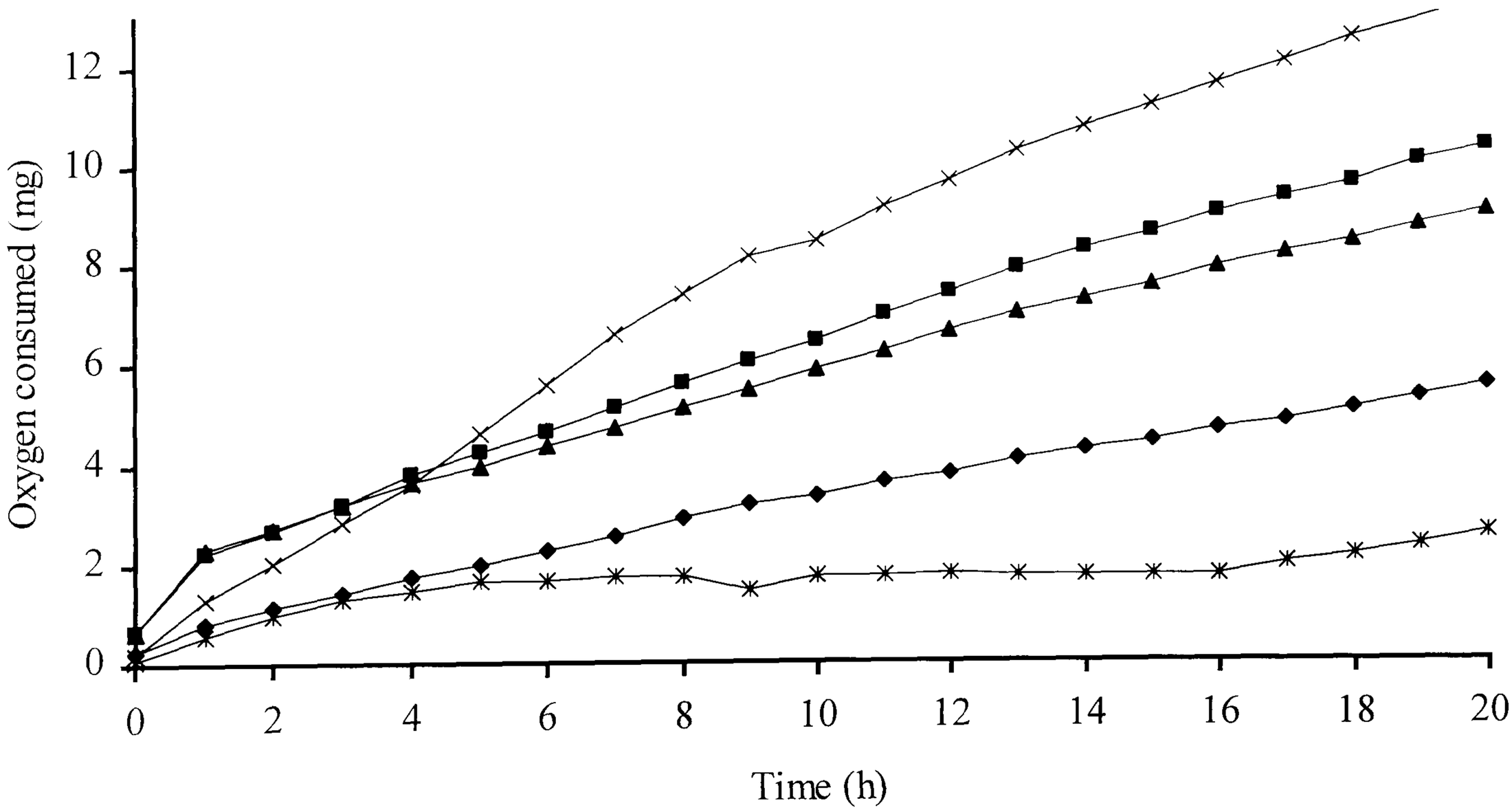

Figure 6.1: Chemical uncoupler effect on oxygen uptake by activated sludge: untreated (✶), trypan blue 18 mg l^{-1} (◆), rotenone 4.0 mg l^{-1} (▲), 2,4 DNP 4.0 mg l^{-1} (■), dicoumarol 0.8 mg l^{-1} (×).

Table 6.1: Rate of oxygen uptake in activated sludge treated with chemical uncouplers (mg O_2 g $MLSS^{-1}$ h^{-1}).

Time (h)	Control (mg O_2 g $MLSS^{-1}$ h^{-1})	2,4 DNP (mg O_2 g $MLSS^{-1}$ h^{-1})	Rotenone (mg O_2 g $MLSS^{-1}$ h^{-1})	Dicoumarol (mg O_2 g $MLSS^{-1}$ h^{-1})	Trypan blue (mg O_2 g $MLSS^{-1}$ h^{-1})
2	0.30	0.68	0.89	0.32	0.55
12	0.08	0.22	0.22	0.21	0.23
20	0.07	0.16	0.17	0.17	0.19

Glucose is a readily metabolisable simple substrate. If glucose is added to activated sludge samples the expectation is for the COD to be rapidly removed as the microorganisms can easily utilise the sugar - the cause of the increased COD. The addition of the inhibitor did not prevent the activated sludge from utilising the additional glucose compared to the control. Considered as COD removal, the inhibitor presence caused a reduction in the removal capabilities of the activated sludge according to the dose applied (Table 6.2). Increasing concentrations of 2,4 DNP decreased the percentage removal of COD. A concentration of 4.0 mg l^{-1} gave the highest increase in oxygen uptake (Figure 6.1) suggesting the greatest extent of uncoupling and resulted in a COD removal of 24-31 % compared to the 37-49 % range of the control. Rotenone and dicoumarol reduced the extent of COD removal further in comparison to 2,4 DNP. In both cases the concentration that produced the most increased oxygen uptake also gave the best COD removal.

Table 6.2: Range of percentage COD removal of activated sludge samples dosed with inhibitor and glucose substrate.

Inhibitor	Dose mgl^{-1}	COD Reduction %
Control	-	37 - 49
2,4 DNP	2.0	30 - 34
	2.5	18 - 23
	3.0	7 - 11
	3.5	13 - 18
	4.0	24 - 31
	6.0	19 - 26
	8.0	14 - 19
Rotenone	2.0	20 - 29
	4.0	23 - 31
	4.5	8 - 15
	5.0	+5 - +15
Dicoumarol	6.8	7 - 16
	8.0	9 - 17
	9.6	12 - 23

More detailed monitoring in bench scale batch tests showed COD removal in both test and control samples were comparable and not statistically different ($F=1.3$, $P<0.05$). The addition of chemical uncoupler either as one initial dose or repetitively on influent addition had little effect the rate of COD removal in activated sludge (Table 6.3). Repetitive dosing of trypan blue and rotenone caused a reduction in the rate of COD removal at 11 d compared to the control. It should be noted that the COD removal rate of the control system increased during the trial period (Table 6.3). This was due to the experimental set up; the activated sludge and settled sewage were collected from different treatment plants and no period of acclimation was occurred prior to the testing. The increase in rate suggests that the activated sludge had at that time become acclimated to the sewage.

Table 6.3: Mean COD removal rate (mg COD g MLSS $^{-1}$ h^{-1}) ± standard deviation in 2,4 DNP and trypan blue and rotenone treated batch-fed activated sludge n=4

		Single Dose		
Day	Control	Trypan Blue	Rotenone	2,4 DNP
2	1.1 ± 0.1	13.8 ± 0.3	1.1 ± 0.1	2.2 ± 0.3
7	2.9 ± 0.7	4.0 ± 0.1	2.9 ± 0.1	2.6 ± 0.1
11	6.3 ± 0.8	12.7 ± 0.5	6.3 ± 0.2	4.1 ± 0.1
		Repetitive Dose		
2	0.2 ± 0.4	4.1 ± 0.1	0.4 ± 0.2	3.1 ± 0.1
7	2.4 ± 0.1	1.9 ± 0.2	2.8 ± 0.1	1.8 ± 0.1
11	16.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	19.4 ± 0.5

The untreated batch test showed a decrease in MLSS across the 11 d. A single dose of all 3 chemical treatments resulted in a reduction in the MLSS greater than that of the control (Table 6.4). With repetitive dosing the reduction in MLSS was much greater; 80% with both rotenone and 2,4 DNP. The MLSS concentration in the trypan blue treated activated sludge was much lower throughout the trial and this may have affected the results obtained.

Table 6.4: Mean percentage change ± standard deviation of MLSS in trypan blue, rotenone and 2,4 DNP treated batch-fed activated sludge - single and repetitively dosed samples n=4

		Single Dose		
Day	Control	Trypan blue	Rotenone	2,4 DNP
Dose mg g MLSS^{-1}		0.01	0.67	0.57
2	7630 ± 244	1280 ± 500	6000 ± 48	7000 ± 133
7	7500 ± 30	840 ± 200	5200 ± 88	5900 ± 124
11	4560 ± 233	620 ± 200	3410 ± 24	3140 ± 116
% removal at 11 d	40	52	44	56
		Repetitive Dose		
Dose mg g MLSS^{-1}		33.0	0.96	1.4
2	7630 ± 244	1320 ± 17	10100 ± 202	6800 ± 136
7	7500 ± 30	860 ± 4	4800 ± 144	6700 ± 73
11	4560 ± 233	760 ± 9	2010 ± 20	1450 ± 58
% removal at 11 d	40	30	80	80

6.4 DISCUSSION

The respirometry experiments showed it was possible to uncouple microbial metabolism by chemical addition. Successful uncoupling was seen as an increase in both amount of oxygen utilised and rate of uptake (Figures 6.1) (Okey and Stensel, 1993; Rich and Yates, 1955).

Okey and Stensel (1993) reported that the response of activated sludge to uncouplers fell into one or more of four categories. The response could be an increase in respiration; reduced synthesis when metabolising growth substrates; reduction in the rate of substrate uptake or toxicity at high concentration ratios. The chemicals tested here exhibited an increase in respiration (Figure 6.1, Table 6.1) and some reduction in the rate of substrate uptake. 2,4 DNP caused the least reduction in substrate utilisation

at low concentrations. Rich and Yates (1955) determined that 2,4 DNP stimulated the removal of the organic fraction of the waste at low concentrations. Rotenone and dicoumarol caused a greater decrease in COD removal. Dicoumarol and 2,4 DNP are oxidative phosphorylation uncouplers acting on one specific part of metabolism - the energetic link that transfers ATP from catabolism to anabolism. Trypan blue is a tri-carboxylic acid cycle inhibitor (part of catabolism) that prevents a specific single reaction of metabolism (Quastel, 1963). Rotenone inhibits nicotinamide adenine dinucleotide (NAD) linked substrate linked oxidation and oxidative phosphorylation (Dawson *et al.*, 1986). There are several paths in metabolism that use NAD linked substrate oxidation and consequently rotenone can exert an effect on a wider proportion of the metabolic paths possibly leading to a greater extent of uncoupling.

The effect of the site of action of the inhibitor was illustrated by the extent of increase of respiration (Figure 6.1). Rotenone generated a higher increase in oxygen consumption than dicoumarol or trypan blue as it had the capability to act on more than one site within metabolism. Although 2,4 DNP is an oxidative phosphorylation inhibitor it is a potent uncoupler at the correct concentrations and here showed the greatest extent of uncoupling. Low and Chase (1996) found dinitrophenol ineffective at metabolic uncoupling and investigated the effect of para-Nitrophenol (4 NP), a stronger uncoupler, on a single culture of *Pseudomonas putida* grown with glucose as the sole carbon source.

One of the chief aims of the activated sludge process is the removal of organic matter or COD. In the bench scale simulation the COD removal capabilities of control, rotenone and trypan blue treated samples were comparable and not statistically different (Table 6.3). Low and Chase (1996) showed inhibition of growth by para-Nitrophenol without an adverse effect on removal of organic matter. Rich and Yates (1955) reported that an increase in substrate uptake occurred at low 2,4 DNP concentrations but at a concentration of greater than 15 mg l^{-1} a decreased rate of assimilation was found.

The MLSS accumulation appeared to decrease with the addition of a single treatment of both rotenone and trypan blue (Table 6.4). Repetitive dosing with trypan blue appeared to increase the reduction of suspended solids further than a single dose. If such a system were to be implemented on a full scale, chemical dosing would likely be at a low rate but continuously with influent to ensure all 'new' microorganisms entering the aeration chamber were treated. The repetitive dosing with influent in the tests appeared to give a greater reduction in biomass production. However, the same regime with rotenone reduced the lowering of biomass.

It is in dispute whether it is the final concentration or the ratio of chemical to MLSS that is of importance for uncoupling. Low and Chase (1996) found the optimum concentration for para-Nitrophenol was 50 mg l^{-1} to achieve uncoupling and a decrease in cell yield. Okey and Stensel (1993) found a minimum concentration of 0.01-0.02 mg uncoupler/mg MLSS was required. With repetitive dosing the concentration of chemical applied varied between 0.001 and 0.03 mg uncoupler/mg MLSS comparable to the range described by Okey and Stensel (1993). In this study addition of chemical uncouplers to the mixed culture of activated sludge caused an increase in oxygen uptake and rate of utilisation but did not prevent the activated sludge from using available substrate (both settled sewage and glucose), nor was the COD removal capacity of the system affected. Treatment with chemical uncouplers resulted in a small reduction of biomass produced in terms of suspended solids.

Uncoupling is a universal system and is not dependent on the microorganism species present. Chemical uncoupling does not necessitate two stage processing or require great oxygen inputs. Further work needs to address the issue of determining precise optimum treatment concentrations and larger scale testing to find the full impact on process efficiency and MLSS yield. At this stage the use of chemical uncouplers has great potential to reduce biomass accumulation without detriment to process efficiency.

6.5 CONCLUSIONS

- The metabolic inhibitors trypan blue, rotenone and 2,4 DNP all caused an increase in oxygen uptake rate suggesting that uncoupling was occurring.
- The rate of COD removal in batch fed chemically treated tests was comparable to and not significantly different from control tests.
- The MLSS of chemically treated activated sludge was reduced in both singly and repetitively dosed batch fed tests. The most significant reductions were achieved with rotenone and 2,4 DNP when dosed regularly with the settled sewage feed; 80% reduction in MLSS occurred with both chemical treatments.
- The successful reduction in MLSS, demonstrated on a small scale, reinforces the suitability of chemical uncouplers for biomass reduction in the activated sludge process.

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Chapter 7: Addition of Rotenone to Activated Sludge – An Attempt at Waste Biomass Reduction

Chapter 7

Addition of Rotenone to Activated Sludge - an Attempt at Waste Biomass Reduction

7.1 INTRODUCTION

The addition of chemical uncouplers has been identified as a possible mechanism for biomass reduction. Rotenone is a widely used uncoupler which selectively inhibits the oxidation of NADH linked substrates, vital in the electron transport mechanism (Horgan and Singer, 1968). Preliminary investigations suggested rotenone to be a strong uncoupler in activated sludge and resulted in a great increase in oxygen uptake rate. Lab scale tests identified that the addition of rotenone caused little impact on COD removal and as such promoted the use of rotenone on a larger scale to determine the effect on biomass yield. This chapter presents the results of a laboratory scale activated sludge process simulation treated with rotenone.

7.2 MATERIALS AND METHODS

A laboratory scale activated sludge simulation was set up using porous pots (Painter and King, 1976). The MLSS were retained within an inner porous section through which the effluent passes (Figure 7.1). The porous pots were seeded with 3 l of activated sludge (Cotton Valley sewage treatment works, Anglian Water Ltd.) An air stone provided both oxygen and mixing within the system. The porous pot had a working volume of 3 l; when fluid levels between the two walls built up to 3 l, effluent passed out of the effluent port. The substrate was pumped directly into the activated sludge. The settled sewage influent was temperature controlled at 20 °C and the porous pots were covered in insulating material in order to maintain a stable operating temperature. The solids were brushed off the inner walls daily. The dissolved oxygen was maintained above 2 mg l^{-1} and the pH between 7 and 8.

A continuous feed of settled sewage was supplied at 0.45 l h^{-1}. Mixed liquor suspended solids (MLSS) were maintained at 2500 mg l^{-1}, the wasted volume being replaced with effluent. The test simulation was treated with 0.2 mg l^{-1} rotenone solution (97 % Sigma Aldrich, Dorset, UK) at a rate of 0.46 l h^{-1} (0.29 mg chemical g $MLSS^{-1}$ d^{-1}) and the control with tap water. Samples of 50 ml activated sludge were investigated for oxygen utilisation using respirometry (Model 017, CES Ltd. Kent, UK). BOD and COD were measured every two days according to standard methods (APHA 1992) and Test and Tube vials (HACH, Camlab, Cambridge, UK).

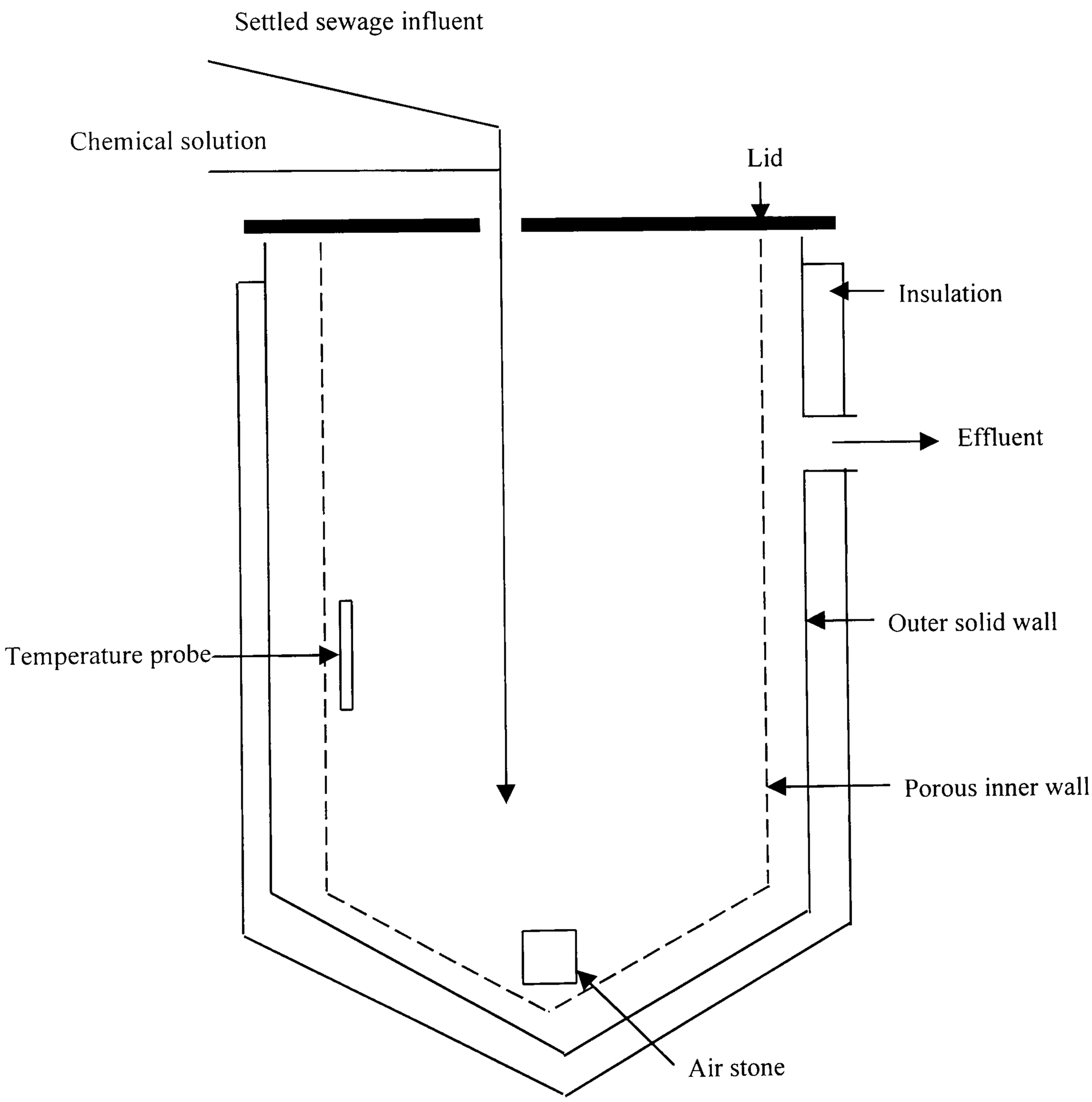

Figure 7.1: Schematic of an activated sludge simulation using porous pot

The effect of the chemical on microbiological diversity was determined microscopically (Model M4000-D, Swift, Japan). Floc shape and size was described using the categories described by Eikelboom and van Buijsen (1981), numbers and types of protozoa were also noted. Floc size was described as small, medium or large in size according to the amount of the field of view they occupied, and as being either open or compact in structure (according to the space between the microorganisms of the floc) and as rounded or irregular in general shape.

Table 7.1: Protozoa species identified microscopically in activated sludge samples

Ciliates	Flagellates	Others
Carchesium	*Bodo*	Amoeba
Epistylis	*Hexamitus*	Thecamoeba
Opercularia	*Monosiga*	Heliozoa
Vorticella	*Pleuromonas*	Rotifers
Aspidisca	*Poteriodeniron*	Nematodes
Belphariama	*Trepamonas*	*Chilodenella*
Colpidium		
Euplotes		
Lionotus		
Paramecium		
Spirostomum		
Trachelophylum		

Approximately 25 species of protozoa were identified, classified into 3 groups (Table 7.1). A species diversity index of the protozoa was calculated using a method adapted from Southwood (1976). This resulted in a single number or species diversity index for the sample under test. The greater the species diversity number the more diverse the sludge in terms of different numbers of species present.

To compare the sets of data generated from the two simulations, statistical tests were carried out. Since the two systems (test and control) were operated in the same way, external factors would have been the same for both; consequently, t-tests were used to compare the means of each parameter for the duration of the trial period the two simulations (Box *et al.*, 1978; Dowdy and Wearden, 1991; Sokal and Rohlf, 1981).

The yield of activated sludge was calculated from the following equation:

$$Y = \frac{MLSS \times Q_w}{BOD_{rem} \times Q} \qquad (7.1)$$

Where Y = The observed yield coefficient (dimensionless)

MLSS = mixed liquor suspended solids (mg l^{-1})

Q_w = waste flow rate (l d^{-1})

BOD_{rem} = BOD removed (mg l^{-1})

Q = influent flow rate (l d^{-1})

As the simulation used porous pots, the effluent suspended solids were negligible and not considered in the yield calculation.

The food to microorganism ratio or rate (F:M) gives an indication of the loading of the system and in conventional activated sludge plants is usually 0.2 – 0.6 (Metcalf and Eddy, 1991). The F:M ratio was calculated from:

$$F{:}M = \frac{Q \times BOD_{inf}}{V \times MLSS} \qquad (7.2)$$

Where F:M = d^{-1}

V = The volume of the reactor (l)

MLSS = mixed liquor suspended solids (mg l^{-1})

BOD_{inf} = influent BOD (mg l^{-1})

Q = influent flow rate (l d^{-1})

The sludge age (θ_c or mean cell residence time) is the length of time in days that a cell is retained in the aeration chamber and was calculated from:

$$\theta_c = \frac{V_r X}{Q_w X_w + Q_e X_e} \qquad (7.3)$$

Where V_r = volume of reactor (l)

X = MLSS concentration in reactor (mg l^{-1})

Q_w = waste flow rate (l d^{-1})

X_w = MLSS concentration in waste flow (l d^{-1})

Q_e = flow rate of effluent (l d^{-1})

X_e = MLSS concentration of effluent (mg l^{-1})

In the simulation, the porous inner wall retained most of the solids with negligible solids passing through to the effluent, consequently to calculate the sludge age, the term Q_eX_e in equation 7. 3 was omitted.

7.3 RESULTS

The mean operating temperature was 11.6 °C in the control and 12.0 °C in the test. The dissolved oxygen was maintained above 2 mg l^{-1}. The average dissolved oxygen concentrations were 2.1 mg l^{-1} and 1.6 mg l^{-1} in the control and rotenone treated simulation respectively. The average pH was 6.9 for both simulations with ranges of 5.8 to 8.4 (Table 7.2).

Table 7.2: Mean simulation operating parameters for untreated and rotenone treated activated sludge

	Control	Rotenone treated
Dissolved oxygen (mg l^{-1})	4.9 ± 3.0	5.3 ± 3.1
pH	6.9 ± 0.7	6.9 ± 0.8
Temperature (°C)	11.6 ± 3.5	12.1 ± 3.5
Sludge age (d)	11.4 ± 1.5	12.9 ± 1.8
F:M (d^{-1})	0.60 ± 0.07	0.61 ± 0.12

Removal of carbonaceous matter was not statistically different between the control and rotenone treated simulations either for COD ($t=0.45$, $P<0.05$) or BOD ($t=1.19$, $P<0.05$) (Table 7.3).

Table 7.3: Concentration and removal of COD and BOD in rotenone treated and untreated activated sludge

BOD	Influent (mg l^{-1})	Effluent control (mg l^{-1})	Effluent rotenone treated (mg l^{-1})	% removal control	% rotenone treated
Pre treatment n= 3	301.3 ± 55.2	18.0 ± 15.3	10.3 ± 5.5	93.3 ± 6.8	96.3 ± 2.6
Post treatment n=12	223.6 ± 102.9	6.6 ± 2.6	7.8 ± 5.5	96.6 ± 1.3	96.0 ± 2.0
COD					
Pre treatment n= 3	377.3 ± 61.0	66.1 ± 30.5	64.1 ± 28.7	82.5 ± 8.2	81.8 ± 10.3
Post treatment n=12	315.6 ± 80.1	25.8 ± 22.7	32.3 ± 32.4	89.7 ± 57.8	89.1 ± 5.9

BOD was successfully reduced by both the treated and untreated activated sludge to the same level about 20 -30 mg l^{-1} (Figure 7.2). The removal capabilities of the simulations were the same both pre and post chemical dosing.

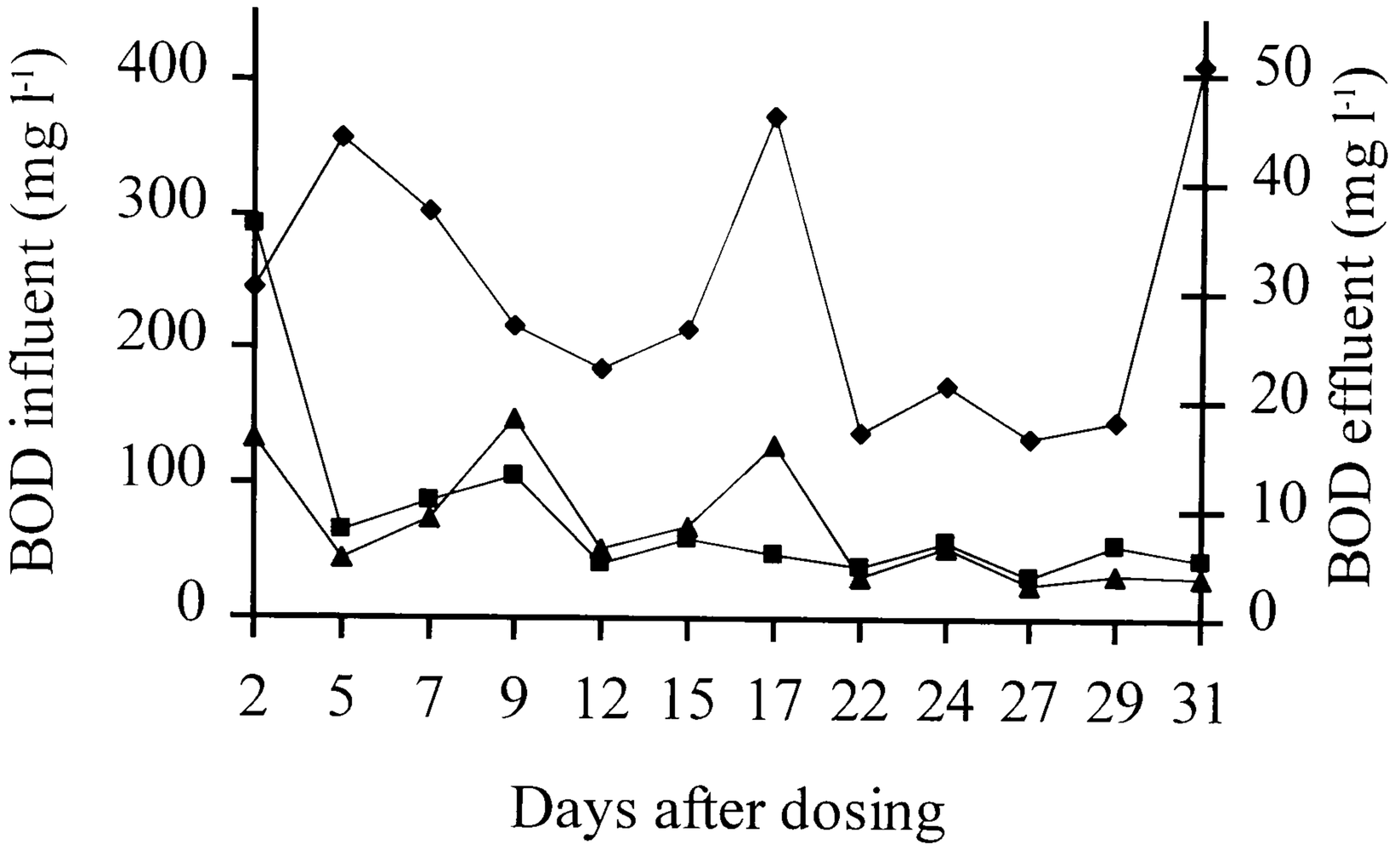

Figure 7.2: Influent (◆) and effluent BOD (mg l^{-1}) of untreated (■) and rotenone treated activated sludge effluent (▲).

The simulations were controlled by maintaining MLSS of the aeration vessels at 2500 mg l^{-1}. The mean sludge ages were 7.8 ± 3.6 d and 7.5 ± 4.8 d before treatment for the control and rotenone simulation respectively. After treatment the mean sludge ages were 11.4 ± 1.5 d and 12.9 ± 1.7 d respectively. This suggested the rotenone treated activated sludge required less solids removal on average to maintain the 2500 mg l^{-1} than the control simulation. Plots of total suspended solids showed that the two simulations were not statistically different ($t=0.44$, $P<0.05$) before chemical dosing. After chemical addition, a decrease in the suspended solids of the treated simulation was seen 7 d after treatment (Figure 7.3). During this period the moving average of TSS in the rotenone treated activated sludge was significantly lower than the untreated ($t= 3.68$ $P< 0.05$). This trend continued until 17 d after chemical addition when the

experiment was terminated. The termination was not due to treatment failure but due to a contamination of the settled sewage which caused cell death in both test and control.

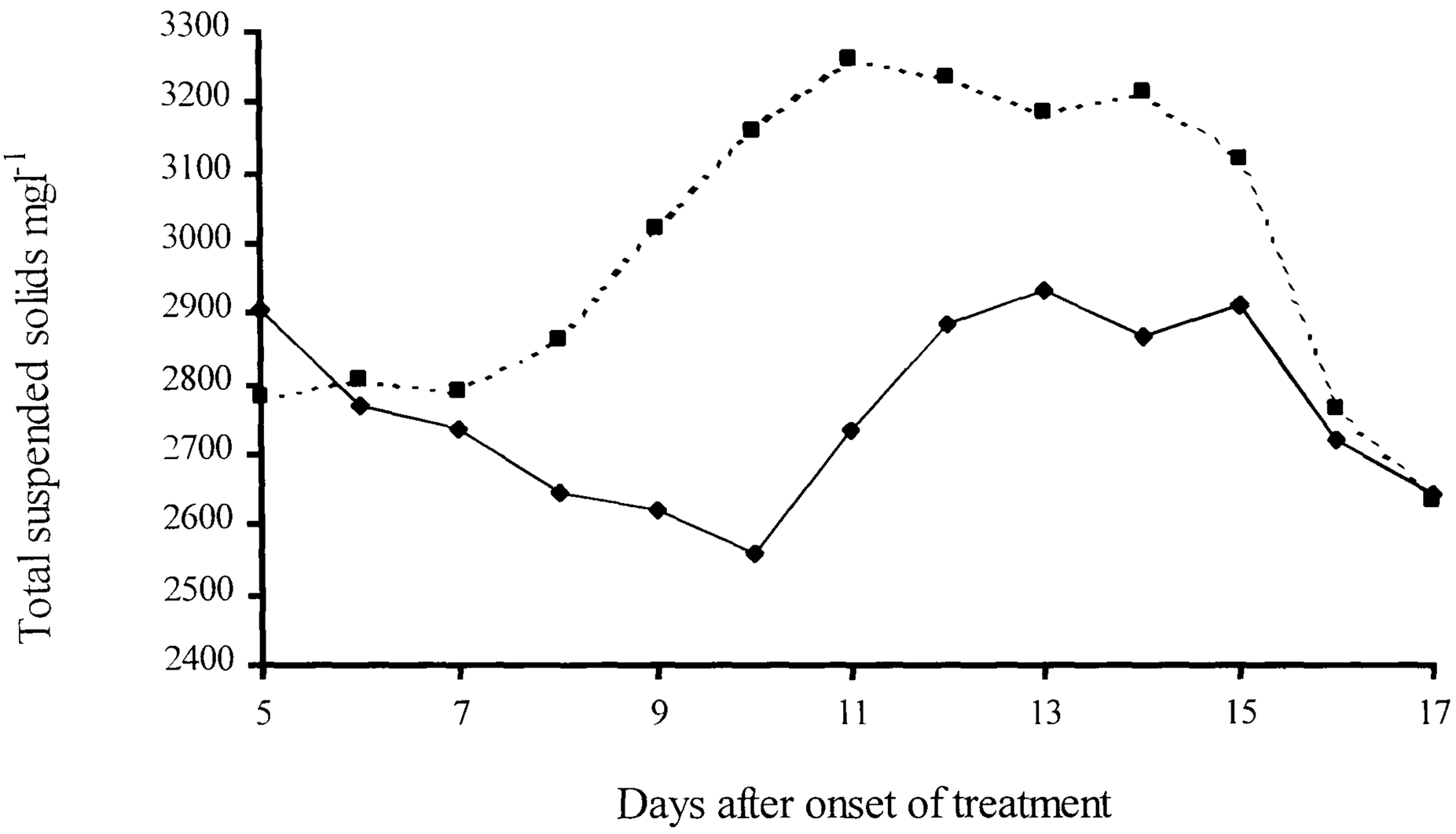

Figure 7.3: 7 day moving average plot of total suspended solids of (■) untreated and (◆) rotenone treated activated sludge

The yield coefficients based on both COD and BOD removal support the trends seen in the TSS (Table 7.4). During the period of chemical treatment an apparent reduction in yield occurred but it was not statistically significant.

Table 7.4: Mean yield coefficients of activated sludge based on COD (Y_{COD}) and BOD (Y_{BOD}) pre and post chemical treatment with rotenone

	Y_{COD}		Y_{BOD}	
	Control	Rotenone treated	Control	Rotenone treated
Pre treatment ± SD n=7	0.38 ± 0.19	0.30 ± 0.17	0.41 ± 0.24	0.33 ± 0.17
Post treatment ± SD n=17	0.33 ± 0.28	0.17 ± 0.15	0.34 ± 0.31	0.28 ± 0.33

The mean number of flocs per sample was lower for the rotenone treated sludge, 15.1 compared to the control with 20.5. For both treatments the majority of the flocs were irregular, compact and either firm or weak in structure. The control also contained flocs that were open in structure whereas the rotenone treated sludge flocs tended to be compact.

Microscopic studies of 24 protozoan species, floc shape and size indicated that the activated sludge composition was not significantly different between the control and rotenone treated samples. Application of a species diversity index adapted from Southwood (1978) showed the activated sludge treated with rotenone was slightly more diverse having an index of 16, the control had an index of 14.5 (Figure 7.4). However the indices were not significantly different ($t=0.51$, $P<0.05$).

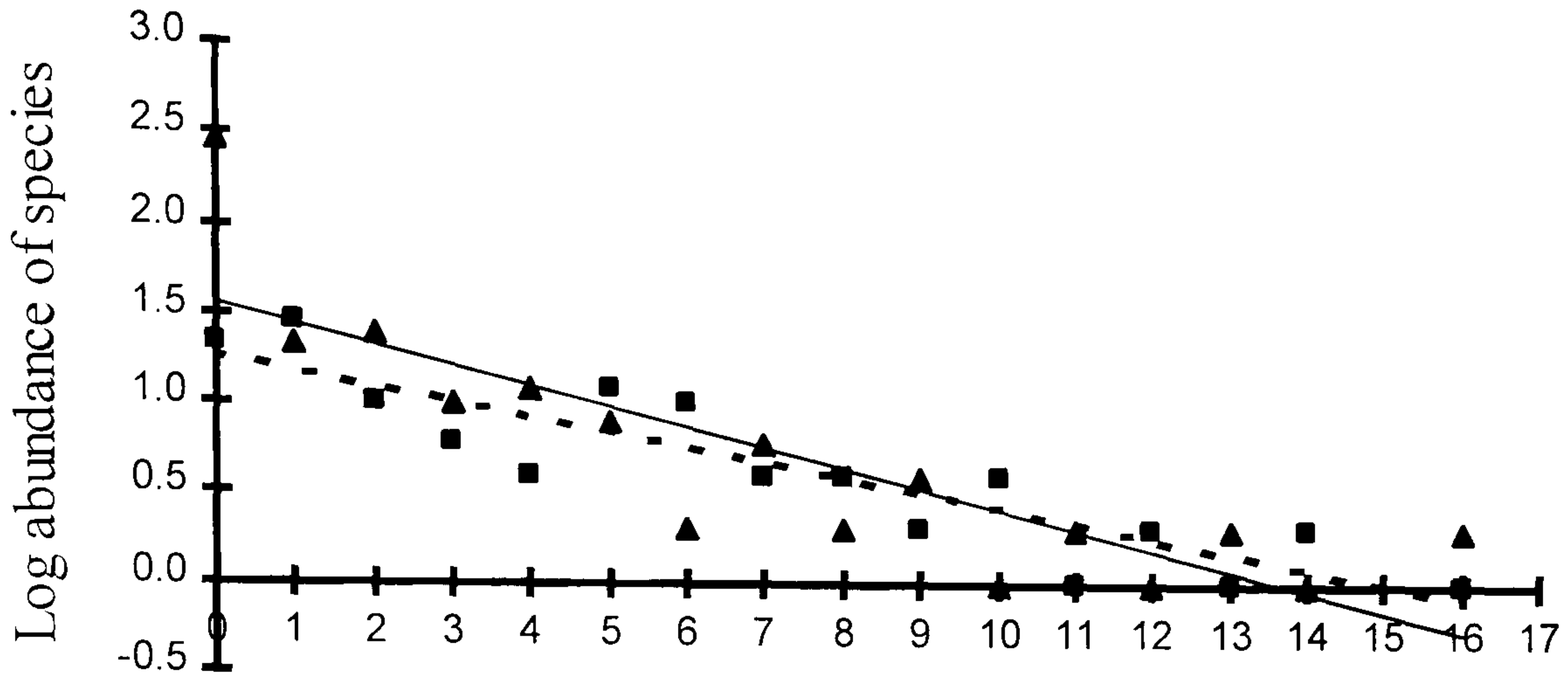

Figure 7.4: Graphical determination of species diversity of untreated (▲ —) and rotenone treated activated sludge (■ – – –).

The amount of oxygen uptake by activated sludge treated with rotenone was greater than that of the control at 10 days after treatment (Figure 7.5). However during the treatment period the mean oxygen uptake rate was not greater for the uncoupler treated sludge, the rates were not significantly different ($t=1.08$, $P<0.05$) the control being 0.049 ± 0.009 and the rotenone treated being 0.040 ± 0.014 mg O_2 mg $MLSS^{-1}$ h^{-1}.

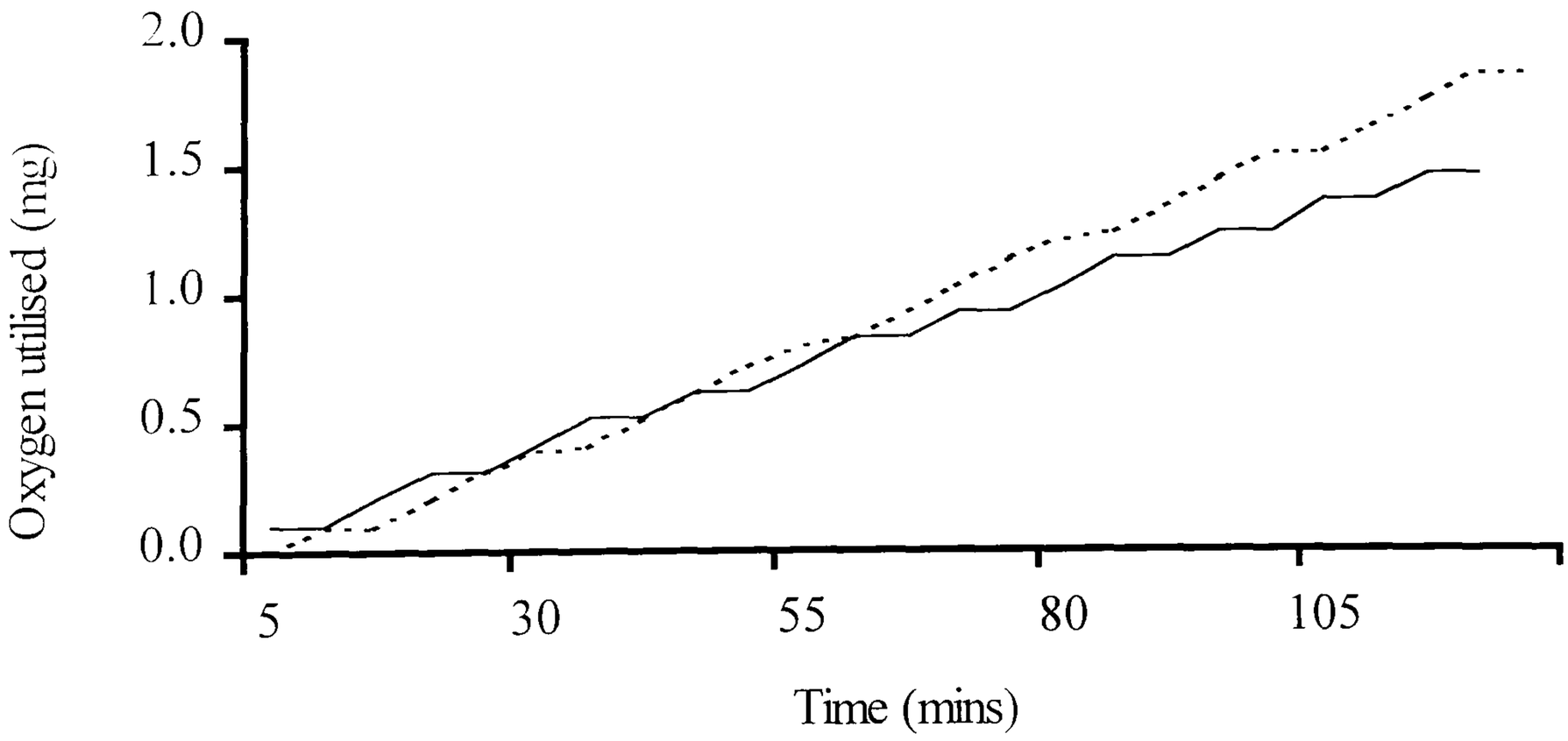

Figure 7.5: Oxygen uptake of activated sludge treated with rotenone (– – – – –) and control (——) 10 days after chemcial treatment.

7.4 DISCUSSION

The simulation was carried out with conditions similar to those of a conventional activated sludge plant. The BOD removal was on average 90%; typically, the average performance of activated sludge plants is around 80% (CIWEM, 1997). The F:M was 0.6 ± 0.07 in the control and 0.61 ± 0.12 in the rotenone treated activated sludge. These were higher than typical values of 0.25 – 0.60 probably due to the high flow rates required (CIWEM, 1997).

The respirometry tests indicated that rotenone was a powerful uncoupler of activated sludge (Chapter 5). However, the oxygen uptake tests were carried out with an absolute concentration of 4 mg l^{-1} rotenone; this was not possible in the activated sludge simulation. Rotenone is only partially soluble in water; up to a value of 0.2 mg l^{-1}, and to achieve continuous concentrations of 4 mg l^{-1} was not feasible.

Data indicated that 50 % inhibition was possible at a concentration of 10 *p*Mol per mg protein (Dawson *et al.,* 1986). The average *E. Coli* cell is approximately 16.2% protein by wet weight (Brock *et al.*, 1984). When considering a concentration of 2500 mg l^{-1} activated sludge and assuming 16.2 % of the weight is protein a concentration of 1.6×10^{-6} mg l^{-1} is required to achieve 50% inhibition. The dose applied in the simulation easily fulfilled this requirement at a loading rate of 0.74 mg l^{-1} d^{-1} rotenone. Rotenone interferes with metabolism by inhibiting electron transport (a fundamental process for chemical energy generation) and NAD linked substrate oxidation (Dawson *et al.,* 1986).

There was a delay between the onset of chemical addition and the first effects seen in MLSS. Experimental work carried out by Horgan and Singer (1968) using rotenone also recorded a pronounced lag in obtaining the maximum inhibition after chemical addition. Analysis showed that a reduction in the amount of MLSS present occurred after 7 d of treatment that was statistically significant, a reduction in the yield was seen concurrently. However over the whole trial period no significant difference was found in yield. This was surprising after the promising effects on oxygen uptake rate in

previous experiments. This was reflected in the oxygen uptake rates measured, which were not as elevated in the simulation as in the respirometry tests.

Microscopic studies determined that there was no difference in species diversity of activated sludge treated with rotenone. Other experimental studies applying the same technique to compare species diversity have found considerable differences when activated sludge was chemically treated: activated sludge treated with an aluminium based polyelectrolyte was more diverse that sludge treated with aluminium sulphate (Clark *et al.*, 1999 in press). It was possible that if enough uncoupler was added to see the increase in oxygen uptake expected then a greater impact on the diversity may have occurred.

There are several techniques available for investigating the diversity of microbial communities. The technique used here used visual analysis and counts of numbers, it was simple and sufficient for the level of detail required. More sophisticated and accurate techniques use 16s ribosomal DNA amplification and comparative analysis of the sequences. Genetic analysis is precise and eliminates the inaccuracies involved in human error in visual identification of species. Such methods are beneficial as it has been found that single activated sludge samples were sufficient for assessing diversity and making inter-plant comparisons (Ballinger *et al.*, 1998; Curtis and Craine, 1998, Liu *et al.*, 1998).

The insolubility of rotenone in water caused practical problems in order to obtain the correct working concentration in the simulation. The results indicated that despite careful calculations to overcome the solubility insufficient rotenone was applied to obtain uncoupling.

7.5 CONCLUSIONS

- The simulation was carried out with conditions similar to those of a full scale works. The addition of rotenone did not have any detrimental effects on the process efficiency in terms of COD or BOD removal.
- The chemical presence had no appreciable effect on species diversity of the protozoa present.
- Despite promising small scale test results, rotenone proved ineffective for biomass reduction in an activated sludge simulation.
- Rotenone was soluble only in very small concentrations and as such was not suitable for a large scale application.

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Chapter 8: The Use Of 2,4 DNP for Biomass Reduction in the Activated Sludge Process

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Chapter 8

The Use of 2,4 DNP for Biomass Reduction in the Activated Sludge Process

8.1 INTRODUCTION

Preliminary studies indicated that uncoupling of microbial metabolism in a mixed culture of activated sludge was possible (Chapter 5). This uncoupling occurred with little or no detriment to pollutant removal in wastewater and a reduced yield of surplus biomass was produced, as also described by Low and Chase (1996). Respirometric analysis of oxygen uptake indicated that rotenone caused the greatest increase in OUR and consequently uncoupling; however this effect was not reflected in an activated sludge simulation. 2,4 DNP gave similar effects to rotenone on a small scale but was more water soluble and so easier to supply in accurate quantities. 2,4 DNP has the potential to cause biomass reduction in activated sludge. This chapter reports on the effect of the chemical on an activated sludge simulation.

8.2 MATERIALS AND METHODS

A laboratory scale activated sludge simulation was set up using porous pots (Painter and King, 1978). The simulations were set up and operated as described in Chapter 7.

Settled sewage from Cranfield University sewage treatment works was supplied at 0.49 l h^{-1} and chemical treatment (or water in the control) at 0.06 lh^{-1} (HRT 5.5 h). The chemical dosed was a 35 mg l^{-1} 2,4-DNP solution; at a rate of 6.7 mg chemical g $MLSS^{-1}$ d^{-1} (Sigma Aldrich Dorset, UK). The MLSS in both pots were maintained at 2500 mg l^{-1} to avoid nematode growth. Dissolved oxygen (Jenway, UK), pH (Hanna Instruments, UK), temperature (RS Components, UK), total and volatile suspended solids of the activated sludge were measured daily. Influent and effluent BOD was measured every 3 d according to standard methods (APHA. 1992).

50 ml samples were analysed respirometrically (Model 017, CES Ltd, Kent UK) every 3 d and then returned to the pots. To compare the sets of data generated from the two simulations statistical tests were carried out. T tests and ANOVA were carried out in order to compare the data (Box *et al.*, 1978; Sokal and Rohlf, 1981).

8.3 RESULTS

The activated sludge simulations were run with conditions as similar to full scale plant operation as possible (Table 8.1). The sludge age of the control before dosing was 1.63 d and of the simulation to be dosed 1.74 d. During the period after dosing the mean weekly sludge age of the control was 1.48 d and of the test dosed with 2,4-DNP 1.50 d. The target MLSS was 2500 mg l^{-1} and maintenance of this resulted in low sludge ages. In the simulation, dissolved oxygen levels were kept above 1 mg l^{-1} to prevent oxygen becoming a limiting factor but as the operating costs were not as important in this case they fluctuated above 4 mg l^{-1} (Table 8.1). pH remained around 7 in both test and control throughout the experimental period (Table 8.1).

Table 8.1: Mean operating parameters of untreated and 2,4 DNP treated activated sludge simulations

	Untreated	2,4 DNP treated
Dissolved oxygen (mg l^{-1})	2.6 ± 1.9	2.3 ± 1.9
pH	6.7 ± 0.4	6.8 ± 0.4
Temperature (°C)	19.9 ± 2.9	19.9 ± 2.9
F:M ratio (d^{-1})	0.19 ± 0.09	0.19 ± 0.10
Sludge age (d)	1.51 ± 0.18	1.54 ± 0.24

Before dosing, the removal of BOD from the control simulation was on average 95.7 ± 2.4% and in the test 94.0 ± 3.9%. After 7 weeks of chemical treatment the mean BOD removal was 94.2 ± 7.5% in the control and 90.5 ± 14.9% when 2,4-DNP treated

(Table 8.2, Figure 8.1). Statistically, after treatment the removal rates were not different (t=2.1, P>0.02).

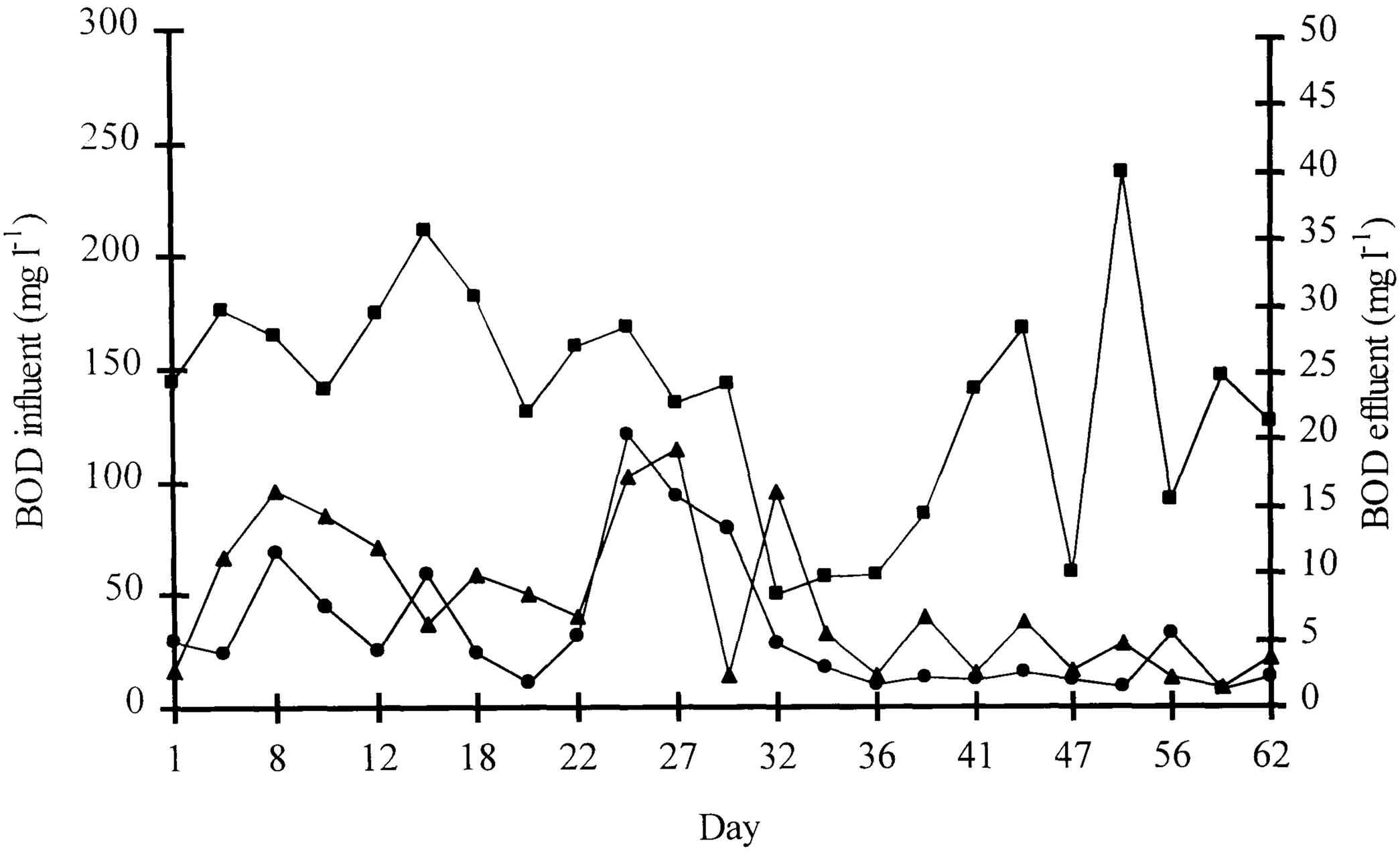

Figure 8.1: Influent (■) and effluent BOD (mg l^{-1}) in untreated (●) and 2,4 DNP treated activated sludge effluent (▲), chemical treatment began at day 4.

Table 8.2: Mean and standard deviations for BOD removal of untreated and 2,4 DNP treated activated sludge

	Influent mg l^{-1}	Effluent control mg l^{-1}	Effluent 2,4 DNP treated mg l^{-1}	% removal control	% removal 2,4 DNP treated
Pre treatment n=3	162 ± 16.3	6.9 ±4.0	10.0 ±6.7	95.7 ±2.4	94.0 ±3.9
Post treatment n=21	128.3 ±58.9	5.3 ±5.2	7.3 ±5.5	94.2 ±7.5	90.5 ±14.9

Throughout dosing with 2,4 DNP the BOD removal of both simulations followed the same trend and remained high, with the exception of days 22 - 37 (Figure 8.1). This lowering in removal corresponded with a rapid increase in the influent temperature. The addition of 2,4 DNP had little effect on process efficiency as measured by BOD removal.

The MLSS was reduced to 2500 mg l^{-1} daily by removal of the requisite volume of solids. It was anticipated that if metabolic uncoupling was successful without significant cell death, then less solids would be removed from the 2,4 DNP treated simulation. The mean weekly yield coefficient before initiation of treatment was greater in the control than the activated sludge to be treated, 0.97 and 0.70 respectively. Once chemical dosing started the test yield dropped to less than the control (Figure 8.2). Indeed, the 2,4 DNP treated activated sludge maintained a lower yield than the control throughout the simulation. The mean yield coefficient after treatment was 0.30 and for the control was 0.42 (Table 8.3). Each of the data points was an average of daily yield values (Figure 8.2, Table 8.3) and as such is summary of an entire week of treatment. The great difference between the test and control in week 8 (Figure 8.2) is a fair and illustrative representation of the yield achieved.

The effluent periodically became yellow; approximately every 5 – 7 d. The colouration was cyclic from clear to yellow to clear and so on. It was possible that some overdosing occurred; as once all the microorganisms had taken up some chemical it then passed through in the effluent. This may have been followed by growth of new microorganisms that absorbed the chemical and no overdosing occurred, so the effluent became clear again. Alternatively, it may be considered that the clear effluent represented stages when the microorganisms became acclimated to the chemical and started to degrade it: upon growth of new biomass degradation ceased.

The chemical 2,4 DNP is yellow in colour as both a solid and as a solution. This yellow colour was reflected in the effluent of the treated activated sludge. As the chemical and effluent were both coloured, samples were analysed with a spectrophotometer at 360 nm in order to quantify the amount of 2,4 DNP released in the effluent (Srivastava *et al.*, 1995). However there were too many interferences in the sewage effluent and the chemical concentration was too low to be accurately measured.

Table 8.3: Mean weekly yield coefficients pre and post treatment of untreated and 2,4 DNP treated activated sludge

	Control	2,4DNP treated
Pre treatment n=2 ± SE	0.70 ± 0.05	0.97 ± 0.04
Post treatment n=7 ± SE	0.42 ± 0.09	0.30 ± 0.05

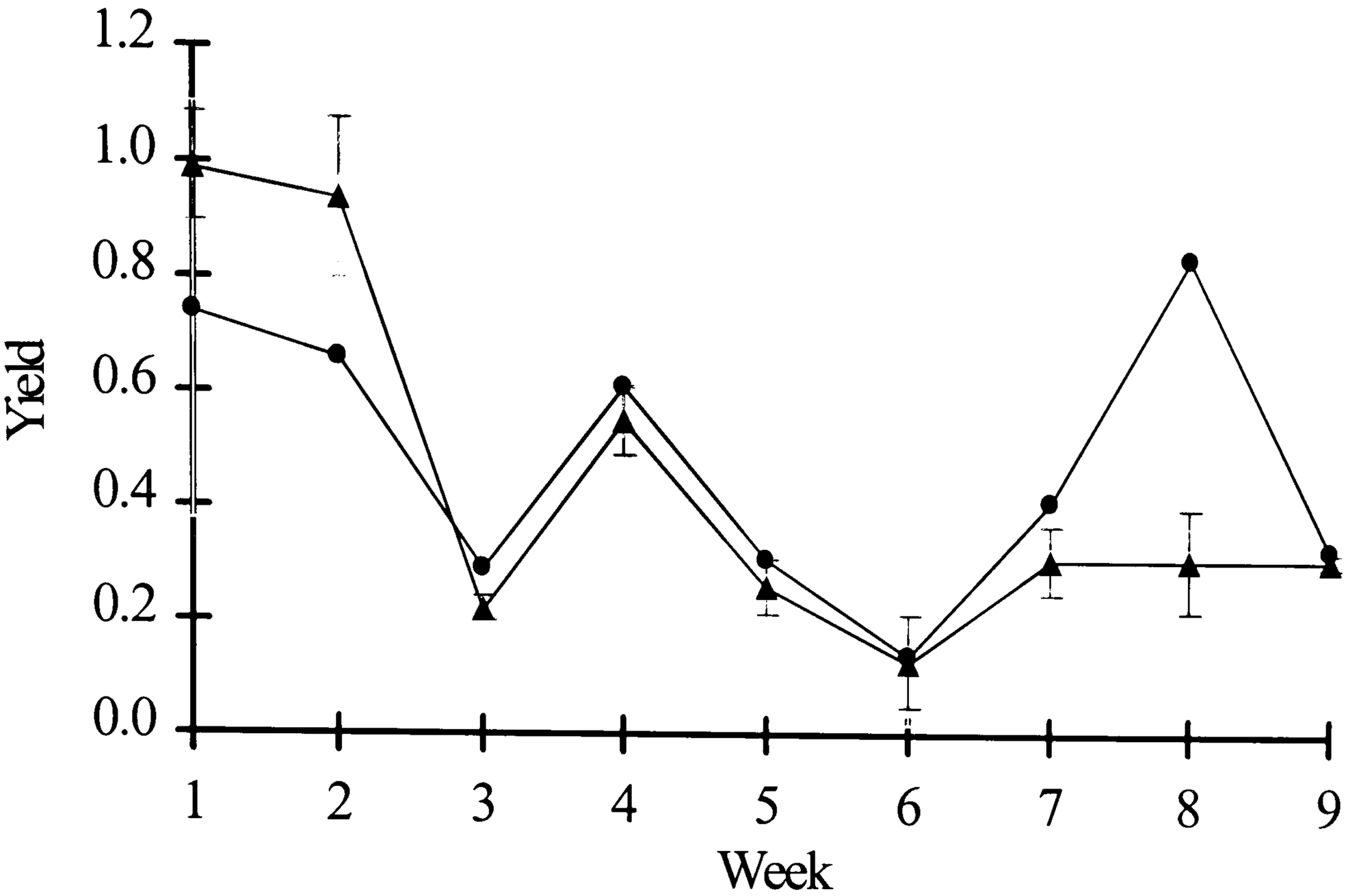

Figure 8.2. Mean yield coefficient of untreated (●) and 2,4 DNP treated activated sludge (▲). Weeks 1 and 2 pre treatment. Error bars represent standard deviations.

Statistically, the yields during the treatment period were compared between the two simulations using an ANOVA to account for the difference between treatment and any variation within the treatment. After treatment with 2,4 DNP the yield of the simulation was significantly reduced from 0.97 $\pm$ 0.04 to 0.30 $\pm$ 0.05 ($t=6.92$, $P>0.01$) whereas there was no difference in yield statistically between the untreated system before and after treatment. After treatment, the range of yield values for the control at 0.14 – 0.84 was greater than that of the 2,4 DNP treated activated sludge, 0.13 - 0.55. That is, the yield was less variable once chemically treated. The mean yield after treatment was significantly lower than the control (ANOVA $F=3.24$. $P>0.1$). The addition of 2,4 DNP significantly reduced the yield coefficient and therefore the biomass production.

8.4 DISCUSSION

Respirometry experiments showed it was possible to uncouple microbial metabolism by chemical addition. Successful uncoupling was seen as an increase in both amount of oxygen utilisation and rate of uptake (Chapter 5). A number of chemicals were good inhibitors. Rich and Yates (1955) found that on a laboratory scale the removal of organic matter by activated sludge was stimulated by 2,4 DNP increasing with concentration up to 25 mg l^{-1}, above this the activity of the activated sludge was slowed. Rich and Yates (1955) investigated the permanency of 2,4 DNP in the mixed liquor and reported that settling and washing with synthetic sewage nullified the effect of the chemical. Such properties made 2,4 DNP a good candidate for biomass reduction. However, Low and Chase (1996) investigated 2,4 DNP for biomass reduction in a single species culture, fed with synthetic sewage, and found it less potent than other chemicals under test.

The 2,4 DNP was continuously dosed at a low rate rather than in one initial dose. This regime is more likely in a full scale system and ensured that all microorganisms in the system received sufficient dosage. Due to its degree of lipophilicity, 2,4 DNP diffuses passively across biological membranes at the physiological pH of 7.4 (Riveranevares *et al.*, 1995). The chemical acts on paths inside the cells; and would eventually be used up on a single dose if further microorganisms enter the aeration chamber. As the chemical needs to act within the cell, a time lag was expected between initiation of chemical treatment and any effects on BOD removal or biomass production. On a daily basis a small lag was observed but considered as a weekly yield no lag was seen. (Figure 8.2).

Maintenance of a MLSS concentration of 2500 mg l^{-1} resulted in low sludge ages due to the rapid growth of the microorganisms. The microorganisms must have sufficient retention time in the reactor to sustain a healthy population. The treatment capability of the system will diminish if the microorganisms have insufficient time in the reactor; in this simulation the treatment capability remained high throughout probably aided by the low F:M or loading rates. Heterotrophic bacteria have fast growth rates and so

short sludge age requirements, the autotrophic bacteria carrying out nitrification require longer sludge ages (CIWEM, 1997). Nitrification was not achieved in this simulation as a consequence of the short sludge age.

The general purpose of the activated sludge process is in the removal of organic matter or BOD. In the simulation the BOD removal capabilities of both control and 2,4 DNP treated activated sludge were comparable and not statistically different (Table 8.2). Results of other studies are conflicting. Rich and Yates (1955) reported that an increase of substrate uptake (organic matter) occurred at low 2,4 DNP concentrations but a decreased rate (that was reversible) was found at concentrations greater than 15 mg l^{-1} after 2.5 h of contact. Okey and Stensel (1993) investigated 2,4 dichlorophenol (2,4 DCP); a powerful uncoupler with the same basic skeleton as 2,4 DNP. At bench scale the presence of uncoupler increased respiration and reduced substrate uptake by a factor of 2 to 5. The general process efficiency when considered as BOD removal was not affected by the addition of the chemical uncoupler in this study.

However, the chemical was only of use if it was capable of lowering biomass production. The yield coefficient gives an indication of the amount of biomass produced in relation to the organic matter removed from the influent. In activated sludge plants the yield coefficient is typically 0.5 (Metcalf and Eddy, 1991). In this study the yield of the treated simulation remained lower throughout the experiment and was statistically significant. Other processes are capable of reducing biomass yield. Ratsak *et al.* (1993) showed a decrease in biomass yield of 12-43 % using a 2 stage cascade culture involving ciliate grazing. However, to obtain such yields it was necessary to identify the predators capable of the most efficient grazing on the biomass available. Uncoupling is a more universal process and is not dependent on the species present. In this study, the yield was greater than that reported for extended aeration processes. Reddy *et al.* (1983) described extended aeration processes capable of zero yields as excess bacteria either died, lysed or were consumed by predators. Although very efficient in yield reduction, extended aeration is in effect two unit processes, biological treatment and aerobic digestion. Consequently energy requirements are

high, especially the oxygen requirement for digestion. Oxic/anoxic cycling can reduce biomass production with yield coefficients of 0.33 (Chudoba *et al.*, 1992). This value is close to that found in this study with 2,4 DNP. Chemical uncoupling has the potential to lower biomass production without additional treatment stages but achieving yields comparable to other systems.

As 2,4 DNP is an organic chemical it is potentially biodegradable and may be used by the microorganisms as an additional carbon source. In the simulation biodegradation did not appear to occur as the yield was lowered suggesting that uncoupling was taking place. A known metabolite of 2,4 DNP is nitrate. Monitoring nitrate production is one method of determining the presence of biodegradation; any further studies should encompass measurement of nitrate concentrations. The multitude of species present in activated sludge continually alters due to new microorganisms coming in the sewage. Although some species may be capable of adaptation to the chemical, the majority may not. No adaptation became apparent for the duration of the simulation.

During the simulation cyclic colouration of the effluent occurred. This supported the idea that no adaptation had occurred. The continuation of colouration in the effluent suggested that should this effluent be discharged then chemical would pass into the receiving water. This may be unacceptable environmentally, however methods are available for removal of 2,4 DNP and will be addressed later (Chapter 11). Dependant on the concentration of chemical in the effluent dilution in the receiving water may result in concentrations below those toxic to the wildlife. It was likely that the system was over dosed with chemical, which contributed to the colouration in the effluent and subsequent application of the system should consider reduced dosing rates.

The difference between the two mean yields after treatment was small: 0.42 control, 0.30 2,4 DNP treated. Such a difference on a real scale is considerable. A full scale plant treating 35,000 m^3 d^{-1} operating with a yield coefficient of 0.42 would produce 1180 t of sludge per year. If the plant operated at a yield of 0.3 as in the treated system described here, the biomass produced would be 843 t per year. Therefore the cost of

treatment and disposal for 337 t would be saved. Uncoupling in activated sludge in two full scale plants in Phoenix, Arizona was reported by Okey and Stensel (1993). A chemical although unknown was classified as an uncoupler. The effects of uncoupling at large scale were an increase in the oxygen requirement, a modest decrease in substrate uptake and a decrease in net synthesis to substantially zero.

8.5 CONCLUSIONS

- The addition of 2,4 DNP to an activated sludge simulation reduced mean biomass yield from 0.42 in a control system to 0.3 in a chemically treated system.
- The reduction in yield was achieved without affecting process efficiency in terms of BOD removal.
- Such a methodology showed promise for reducing biomass yields in full scale activated sludge plants.

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Chapter 9: The Use Of 4 – Nitrophenol for Biomass Reduction in the Activated Sludge Process

Chapter 9

The Use of 4-Nitrophenol for Biomass Reduction in the Activated Sludge Process

9.1 INTRODUCTION

Initial oxygen uptake rate studies suggested that rotenone and 2,4 DNP resulted in the greatest amount of uncoupling in activated sludge (Chapter 5). Laboratory scale studies demonstrated that rotenone was unsuccessful for biomass reduction but that 2,4 DNP lowered the activated sludge yield. The yield was decreased from 0.42 in the control to 0.30 in the biomass treated with 2,4 DNP. 4 nitrophenol or para nitrophenol is a far more powerful uncoupler than 2,4 DNP, and trials with a *Psuedomonas* strain gave reductions of up to 62 % in yield (Low and Chase, 1998). Addition of 100 mg l^{-1} of 4 NP reduced the yield of bacteria from 0.63 to 0.23 with a simultaneous increase in specific substrate uptake rate using glucose substrate and a single strain of bacteria (Low and Chase, 1998). This chapter investigates the effect of 4 NP in an activated sludge simulation.

9.2 MATERIALS AND METHODS

An activated sludge simulation was set up using mixed liquor from Cotton Valley sewage treatment works (Anglian Water Ltd) (Painter and King, 1978). The operating conditions were the same as detailed in Chapter 7.

A continuous feed of settled sewage (Cranfield Sewage treatment works) at 0.437 l h^{-1} and treatment of water or 4 NP (0.1 mg l^{-1}) at 1 ml min^{-1} were supplied (a rate of 0.02 mg chemical g $MLSS^{-1}$ d^{-1}). Dissolved oxygen (Jenway, UK), pH (Hanna Instruments, UK), temperature (RS Components, UK), were monitored daily. Every 3 days, MLSS and BOD were measured according to standard methods (APHA 1992) and COD by Test and Tube vials (HACH, Camlab, Cambridge, UK). 50 ml respirometric samples

were investigated every three days to look at oxygen uptake and then returned to the simulation (Aerobic Respirometer model No. 017, CES ltd. Kent, UK).

Species diversity was monitored microscopically along with floc size and morphology. Species diversity indices were calculated using a method adapted from Southwood (1978). Floc morphology was described according to the categories stated by Eikelboom and van Buijsen (1981). Flocs were categorised as being small, medium or large in size according to the amount of the field of view covered, open or compact in structure and rounded or irregular in shape (Appendix 2). Numbers of flocs and protozoa per ml determined using a deep cell counting chamber (Improved Neubauer Heamocytometer, Weber, UK).

Statistical tests were used to aid comparison of the data; ANOVA and t-tests were used to compare the means of each parameter for the duration of the trial period between the two simulations (Box *et al.*, 1978; Dowdy and Wearden 1991; Sokal and Rohlf, 1981).

9.3 RESULTS

The simulations were carried out with conditions approximating full scale operation as far as possible (Table 9.1). The activated sludge simulation was operated by maintenance of suspended solids at 2500 mg l^{-1}. Food to microorganism ratio was less than that expected in a full scale plant but remained consistent throughout the trial (Table 9.1). Respirometric analysis of samples for oxygen uptake rate showed that addition on the 4 NP increased the rate of uptake of the biomass suggesting that uncoupling occurred (Table 9.2).

Table 9.1: General mean operating parameters of the activated sludge simulation chemically treated with 4 NP

	Control	4 NP treated
Dissolved oxygen (mg l^{-1})	3.7 ± 2.3	4.4 ± 2.4
pH	7.1 ± 0.3	7.1 ± 0.2
Mean temperature (°C)	17.0 ± 1.2	16.7 ± 1.3
Minimum temperature (°C)	16.0 ± 1.1	15.7 ± 1.2
Maximum temperature (°C)	20.6 ± 2.3	20.2 ± 2.4
F:M ratio (d^{-1})	0.24 ± 0.09	0.27 ± 0.12
Sludge age (d)	10.8 ± 4.8	12.4 ± 4.0

Table 9.2: Rate of oxygen uptake of untreated and 4 NP treated activated sludge in mg O_2 mg $MLSS^{-1}$ h^{-1}

	Control	4 NP treated
Pre treatment ± SD	0.14 ± 0.01	0.14 ± 0.02
Post treatment ± SD	0.25 ± 0.08	0.36 ± 0.10

Both BOD and COD removal were high throughout the simulation (Figure 9.1 and 9.2). BOD removal was greater than 93% and COD removal greater than 84% across the duration of the trial (Table 9.3). Pre and post 4 NP treatment there was no statistical difference between the test and control samples for BOD removal ($t= 0.47$, $P<0.05$). After chemical treatment the removal of COD was comparable between test and control; statistically a small decrease was seen in the sample treated with 4 NP ($t=2.68$, $P<0.05$).

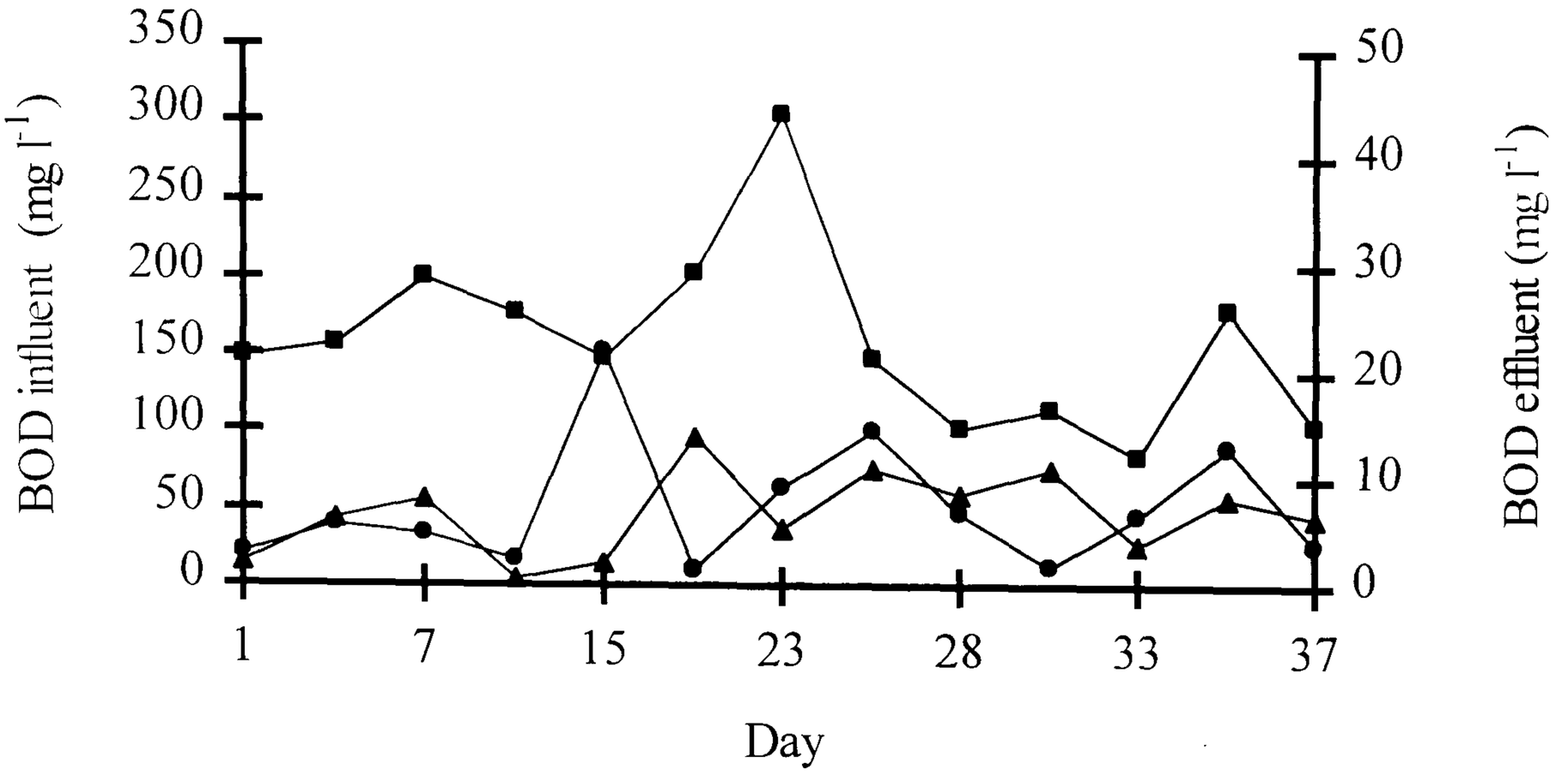

Figure 9.1: Influent (■) and effluent BOD (mg l^{-1}) of untreated (●) and 4 NP (▲) treated activated sludge effluent (treatment began on day 11)

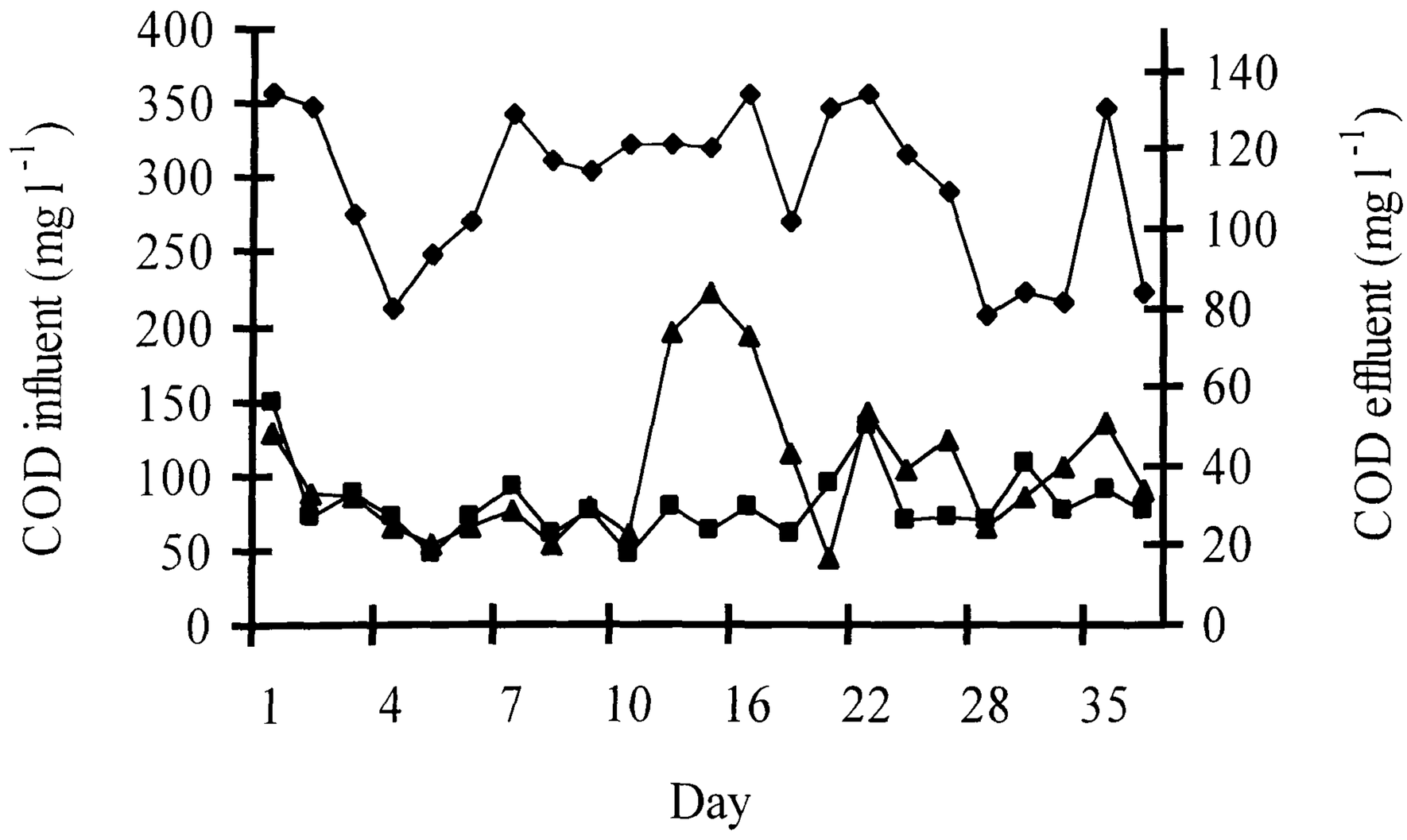

Figure 9.2: Influent (◆) and effluent COD (mg l^{-1}) of untreated (■) and 4 NP (▲) treated activated sludge effluent (treatment began on day 11)

Table 9.3: BOD and COD removal of activated sludge pre and post chemical treatment with 4 NP

	% BOD removal		% COD removal	
	control	4 NP treated	control	4 NP treated
Pre treatment ± SD	97.5 ± 0.9	97.7 ± 0.9	90.2 ± 2.1	90.5 ± 2.2
Post treatment ± SD	94.6 ± 2.7	93.7 ± 4.0	89.0 ± 3.1	84.5 ± 5.1

The yields of biomass production for both the control and chemical treated activated sludge were variable throughout the simulation (Table 9.4). Prior to treatment there was no significant difference between the yields, they appeared to have been drawn from samples with the same mean. After chemical treatment started a decrease in the mean weekly yield was seen (Figure 9.3) which continued for the duration of the test. The yield of 4 NP treated activated sludge was statistically lower than that of the untreated for the treatment period ($t=1.21$, $P<0.10$).

The yield of the 4 NP treated activated sludge fluctuated on a daily basis. This fluctuation coincided with changes in the effluent colour. When dissolved in water 4 NP was yellow in colour. The effluent from the chemically treated simulation became coloured yellow in a cyclic fashion; from clear to yellow to clear and so on. The appearance of yellow colour in the effluent corresponded to times when the yield was much lower than the control. When the effluent was clear, the yield was lower than the control but not by as great an extent. It was this trend that created such variability about the mean yield values obtained. When the effluent was clear there was possibly some degradation of the chemical presumably by acclimated microorganisms. Such cyclic nature in the system may have implications in the rest of process performance. Such a phenomenon may be detrimental as the treatment may not be consistent and consequently the effluent quality.

Table 9.4: Yield of activated sludge in terms of both BOD and COD removal in untreated and 4 NP treated samples

	Y_{BOD}		Y_{COD}	
	control	4 NP treated	control	4 NP treated
Pre treatment ± SD	0.54 ± 0.68	0.44 ± 0.69	0.38 ± 0.48	0.16 ± 0.24
Post treatment ± SD	0.44 ± 0.69	0.19 ± 0.36	0.31 ± 0.41	0.11 ± 0.20

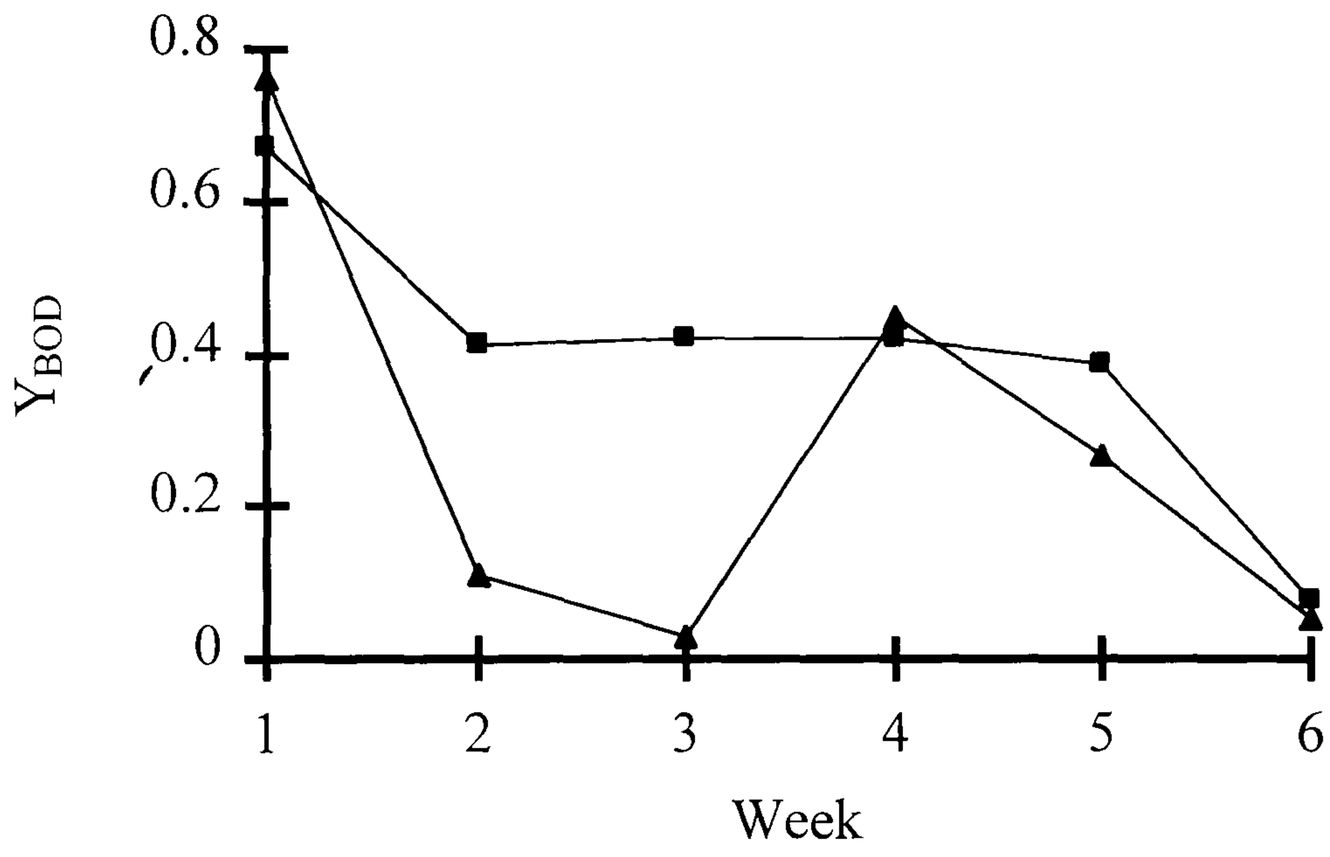

Figure 9.3: Yield of biomass in untreated (■) and 4 NP (▲) treated activated sludge. Week 1 pre treatment and weeks 2 - 6 post treatment.

The species diversity indices of the untreated and 4 NP treated activated sludge were 16.9 and 17.7 respectively. This indicated that the treated sludge was a little more diverse, that is there were a greater number of different species. Statistics suggest that the mean total numbers of occurrence were not significantly different ($t=0.12$, $P<0.05$) but that the numbers of each species occurring were different between the treated and control ($F=24.6$ $P<0.05$).

The addition of the chemical caused visual differences in the floc size and shape. Floc morphology was classified by 6 classes (Table 9.5), in the untreated sludge the flocs fell into only 2 of these categories and generally the flocs were larger and so fewer numbers found per ml. The treatment of 4 NP displayed flocs that were more variable and all 6 classes were seen. Much greater numbers occurred per ml as the flocs were considerably smaller.

Table 9.5: Mean numbers of flocs per ml described by shape, of untreated and 4 NP treated activated sludge

	Number of flocs per ml x1000	
Floc shape	Control	4 NP treated
Firm,round,open	0	73
Firm,round,compact	52	67
Firm,irregular,open	0	0
Firm,irregular,compact	42	75
Weak,round,compact	0	20
Weak,irregular,compact	0	50
Total	94	215

9.4 DISCUSSION

An increase in the oxygen uptake rate from 0.25 to 0.36 mg O_2 mg $MLSS^{-1}$ h^{-1} was seen in the 4 NP treated sludge that indicated that some uncoupling was occurring. This increase in oxygen utilisation has been found in several organisms treated with 4 NP. Clowes *et al.* (1950) reported an increase in oxygen uptake and an inhibition of phosphorylation in cell free systems of arbacia eggs with several substituted nitrophenols. The main aim of the activated sludge process is in removal of carbonaceous matter. Low and Chase (1996) documented an increase of 50% in the substrate degradation rate when a culture of *Pseudomonas* was treated with 50 mg l^{-1}.

The activated sludge simulation carried out did not measure specific substrate uptake rate. However, no detriment to carbonaceous substrate removal was seen in terms of BOD when activated sludge was treated with 4 NP. A small reduction in the COD removal occurred in the 4 NP treated biomass.

The yield of biomass produced was reduced by the addition of 4 NP from 0.44 to 0.19 (Y_{BOD}) and from 0.31 to 0.11 (Y_{COD}). The pre and post yield figures were significantly different, however on a daily basis a lot of fluctuation occurred in the yield of both the control and chemically treated activated sludge (which was reflected in the standard deviation of the yield figures). On a daily basis the yield of the treated simulation was lower than that of the control.

Low and Chase (1998) reported even greater yield reductions than found in this study. A true growth yield of 0.632 ± 0.031 in a *Pseudomonas* strain was lowered to only 0.228 ± 0.008 treated with 100 mg l^{-1} 4 NP. Low and Chase (1996) reported that it was the absolute concentration of 4 NP that was important not the loading rate. The simulation carried out here used a loading rate of 0.02 mg g^{-1} d^{-1}, this was calculated from initial respirometric testing in which the optimum concentration for biomass reduction without detriment to COD removal was 50 mg l^{-1}. However, if this loading is considered as part of the influent flow, a concentration of only 12 mg l^{-1} was achieved, which may explain the yields being less than those reported elsewhere. The reported increase in the specific rate of substrate uptake suggested the possibility of increasing loads to existing plants or reconsidering volumes required when planning new facilities. The small reduction in COD removal that occurred during the end of the trial period indicated that such an increase in rate of substrate removal may ultimately result in lower overall removal efficiencies.

Investigations into the effect of 4 NP addition on maintenance energy requirement showed that it remained fairly constant irrelevant of inhibitor concentration suggesting that with a lowered ATP availability the microorganisms meet the maintenance energy requirements before utilising energy for anabolism (Low and Chase, 1998). There was

low substrate availability in this study which was reflected by the low F:M ratios. The low F:M may have resulted in the fluctuating yields. Reducing F:M is a standard method for reducing biomass accumulation, since the maintenance energy requirement would be satisfied first and subsequently, anabolic reactions allowing growth take place. If insufficient substrate was available in both test and control then growth would only happen once the maintenance energy had been met hence inconsistent growth patterns resulting in variable yield. The lack of availability of substrate at various stages may account for the possible degradation of the chemical indicated by the lack of colour in the effluent.

The pH of the activated sludge simulations was on average 7.1 in both test and control. 4 nitrophenol is an organic protonphore the action of which is favoured by acidic conditions. Investigations into the effect of pH on the biomass reducing capacity of 4 NP indicated that lowering the pH from 7 had little effect except to further increase the specific substrate uptake rate (Low and Chase, 1998). Studies involving the degradation of nitrophenols by *Pseudomonas putida* stated that the degradation was pH dependant and at pH 7 there was no degradation and at a pH of 6 - 6.5 the chemical degradation was rapid (Zeyer and Kearney, 1984). Of the 3 nitrophenol isomers, the strain of *Pseudomonas* isolated from soil was incapable of degrading para NP or 4 NP at all which makes its selection as an activated sludge uncoupler more suitable (Zeyer and Kearney, 1984). This indicated that at the pH of the activated sludge simulation in this study, little or no degradation of the chemical was likely, favouring uncoupling rather than biodegradation and supply of a further substrate.

This study did not observe nitrification. Studies involving study of effect on nitrification have the capacity to investigate how much if any chemical is broken down and consequently if adaptation to the uncoupler is possible and whether this overrides the biomass reduction. The two enzymatic pathways reported for the degradation of nitrophenol substituents involve either an initial reduction by nitro-reductase causing the release of ammonium or the direct removal of nitrate from the aromatic core causing an increase in the nitrate levels present (Simpson and Evans, 1953).

Identification of increasing ammonia or nitrate levels in the effluent may indicate biodegradation.

Matsui *et al.* (1994) investigated acclimated sludge in continuous and batch modes to determine degradation of 4 NP. They reported operational mode resulted in two very different kinetic patterns (batch fitting Monod kinetics and continuous fitting Haldane) and that the type of 4 NP degrading organism found was different, those in batch having lower affinity and those in continuous mode having a high affinity and were sensitive to the 4 NP. Such a selection in mode for type of microorganism may account for the different yields seen between this study and that of Low and Chase (1998) as they were run in different modes as well as with different microorganisms and influent substrate. The chemical had little effect on the species diversity of the activated sludge in terms of the protozoa and larger organisms present. Addition of chemical resulted in flocs that were much smaller than those in the control simulation. This change in characteristic could prove detrimental to the settling and dewatering properties of the activated sludge. If the solids do not settle as well then carry over to the clarified effluent could occur resulting in poor quality effluent.

9.5 CONCLUSIONS

- The addition of 4 NP to activated sludge fed with settled sewage did not affect process performance in terms of BOD removal. However a small reduction in COD removal did occur.
- Differences in the floc shape and size occurred which may be detrimental.
- The yield coefficient was lowered by the presence of the uncoupler and the system has the potential to reduce biomass production on a larger scale. However due to the possible reduction in effluent quality as a result of altered floc size 4 NP was considered less suitable than 2,4 DNP.

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Chapter 10: Metabolic Uncouplers in Activated Sludge – A Pilot Study Using 2,4 DNP

Chapter 10

Metabolic Uncouplers in Activated Sludge - A Pilot Study Using 2,4 DNP

10.1 INTRODUCTION

2,4 dinitrophenol was identified as a suitable inhibitor for biomass reduction as it reduced yield without detriment to COD removal or nitrification ability in activated sludge (Chapters 5 and 8). So far research has been at laboratory scale with small reactor volumes. For such technology to be implemented at full scale treatment plants the actual effect at a larger scale needs to be quantified.

Despite much research into the effect of metabolic inhibitors on both pure cultures and activated sludge, no pilot trials have been carried out (Low and Chase, 1996; Okey and Stensel, 1993; Rich and Yates, 1955). Pilot scale rigs have been widely used to evaluate research at a larger scale in order to determine the suitability of the technology for full scale application.

The diversity of the size and type of activated sludge pilot plants reported in literature reflects the variety of applications of activated sludge for wastewater treatment. The size of pilot scale work and the type of waste treated can vary widely from 62 l to 1000 l (found in literature) and from a specific synthetic sewage to domestic or industrial sewage (Chiemchaisri *et al.*, 1992; Rensink *et al.*, 1988). The tank configurations often vary, especially when trying to evaluate process alterations. These include pilot plants of 10 m deep activated sludge tanks as a small footprint alternative to conventional activated sludge (trailed in Germany Gnirss *et al.*, 1996). A successful pilot scale investigation of the use of metazoa to reduce yield was carried out resulting in a reduction from 0.4 to 0.15 (Rensink and Rulkens, 1997). One beneficial use of chemical uncouplers is the simplicity with which the treatment can be retrofitted to existing systems. The pilot scale trial carried out with 2,4 DNP was intended to

represent the conventional type activated sludge process which is generally in operation throughout the UK for wastewater treatment. Consequently the system consisted of a single unpartitioned aeration chamber, mixed and aerated via compressed air and standard type settlement tank.

10.2 MATERIALS AND METHODS

A pilot scale plant was constructed at Cranfield Sewage Treatment Works with two identical parallel units, one as a control and one to be treated with the uncoupler (Appendix 1). The operating volume was a 330 l aeration tank. Settling tanks were filled by overflow from the aeration tanks. The return activated sludge was pumped back from the base of the settling tank to the aeration tank. Scrapers in the settling tanks were operated for 10 min every 30 min at 2 rpm. A feed of settled sewage (Cranfield Sewage Treatment Works) was supplied at 950 l d^{-1}, the return activated sludge at a ratio of 1.36 (1296 l d^{-1}) to the influent.

Both the feed and the return sludge fed into a 10 l anoxic zone prior to returning to the aeration tank to reduce the numbers of filamentous bacteria (Figure 10.1). The waste sludge was pumped from the aeration tank for 10 min in every 3 h to give a total waste of 36 l d^{-1}. Waste sludge was collected in a container to check exact volumes wasted before disposal. 2,4 DNP (0.5 g l^{-1}) (Analytical grade, 98% pure, Sigma Aldrich, Dorset, UK) was dissolved in tap water and pumped directly into the aeration tank at a rate of 3.6 ml min^{-1} (a total of 5.2 l d^{-1}) a rate equivalent to 3.15 mg 2,4 DNP g $MLSS^{-1}$ d^{-1}. Water was supplied at the same rate as the chemical to the control channel.

Total suspended solids of the influent, control and test aeration tanks and effluent were measured everyday according to standard methods (APHA 1992). Total solids and total volatile solids of the activated sludge were measured every week according to standard methods (APHA 1992). Ammonia and nitrate of influent and test and control effluents were measured using test and tube vials (HACH, Camlab, Cambridge, UK), nitrite (of effluent) and alkalinity of the influent were determined using standard

methods. SVI was monitored regularly using an Imhoff cone and CST using standard methods (APHA 1992). Samples of activated sludge (50 ml) were measured in a respirometer for oxygen uptake (Model 017, CES Ltd. Kent, UK).

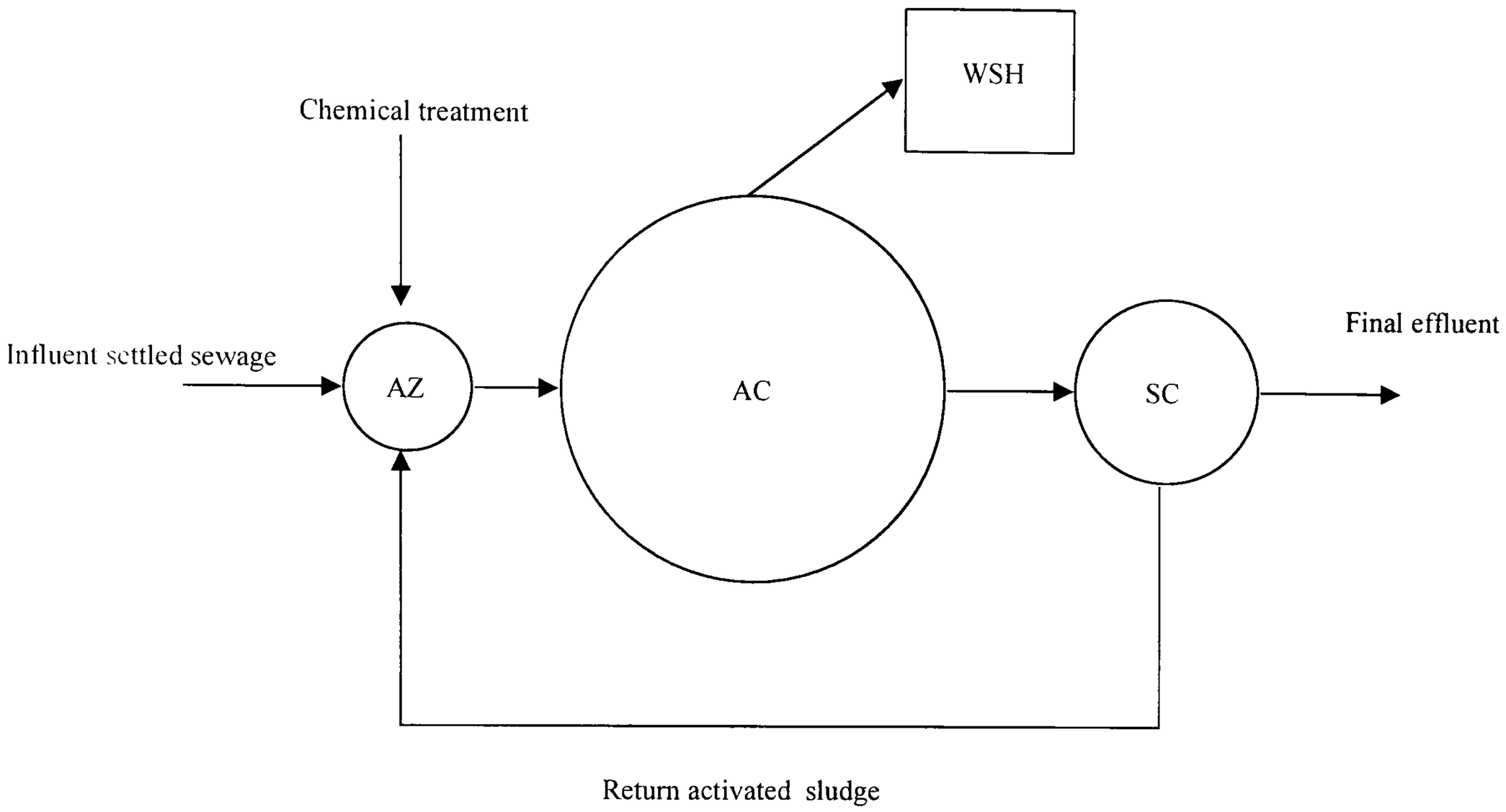

Figure 10.1: Pilot plant process flow diagram (AZ – anoxic zone, AC – aeration chamber, SC – settling column, WSH – waste sludge holding tank

Species diversity was determined microscopically (Swift, model M4000-D, Japan) along with floc size and morphology. Species diversity indices were calculated using a method adapted from Southwood (1978) (See chapter 7 for full details). Floc morphology was described according to the categories stated by Eikelboom and van Buijsen (1981). Flocs were categorised as being small, medium or large in size according to the amount of the field of view covered, open or compact in structure and rounded or irregular in shape (Appendix 2). Numbers of flocs and protozoa per ml determined using a deep cell counting chamber (Improved Neubauer Heamocytometer, Weber, England).

The yields of the two pilot units were calculated from:

$$Y_{BOD} = \frac{(X_w Q_w) + (X_e Q_e)}{BOD_{REM} Q} \qquad (10.1)$$

Where Y_{BOD} = the observed yield coefficinet in terms of BOD (mg MLSS mg BOD^{-1})

Q_w = waste activated slduge flow rate (l d^{-1})

X_w = MLSS of waste sludge (mg l^{-1})

Q_e = effluent flow rate (l d^{-1})

X_e = effluent MLSS (mg l^{-1})

BOD_{REM} = BOD removed (mg l^{-1})

Q = influent flow rate (l d^{-1})

Statistical tests were used to aid comparison of the data; ANOVA and t-tests were used to compare the means of each parameter for the duration of the trial period the two simulations (Box *et al.*, 1978; Dowdy and Wearden, 1991; Sokal and Rohlf, 1981).

10.3 RESULTS

The pilot operating conditions were similar to those of a full scale wastewater treatment plant (Table 10.1). The mean weekly sludge ages of the activated sludge decreased after treatment in both control and test plants: 6.8 ± 2.3 d pre and 5.3 ± 0.3 d post treatment in the control stream and 7.9 ± 2.9 d pre and 5.4 ± 1.2 d post 2,4 DNP treatment, in all cases the sludge age was sufficient to support nitrification. A precise volume of sludge was wasted daily, however the sludge age did not remain fixed as the effluent suspended solids varied during the trial. The mean pH of the influent settled sewage was 7.4 ± 0.2, and the mean pH of the two effluent streams were very similar (Table 10.1).

Table 10.1: Mean operation parameters of the activated sludge pilot plant

	Control	2,4 DNP treated
Dissolved oxygen, (mg l^{-1})	4.7 ± 1.9	4.7 ± 2.7
pH	6.4 ± 0.3	6.2 ± 0.5
Mean temperature, (°C)	17.2 ± 1.2	16.9 ± 1.5
F:M (d^{-1})	0.21 ± 0.09	0.25 ± 0.26
Sludge age, (d)	6.1 ± 1.3	6.7 ± 2.0

The mean BOD in the effluent from the control was 16.2 mg l^{-1} and 18.4 mg l^{-1} in the 2,4 DNP treated effluent (Figure 10.2). The mean COD in the effluent for the control was 23.4 mg l^{-1} and 33.7 mg l^{-1} in the 2,4 DNP treated effluent (Figure 10.3).

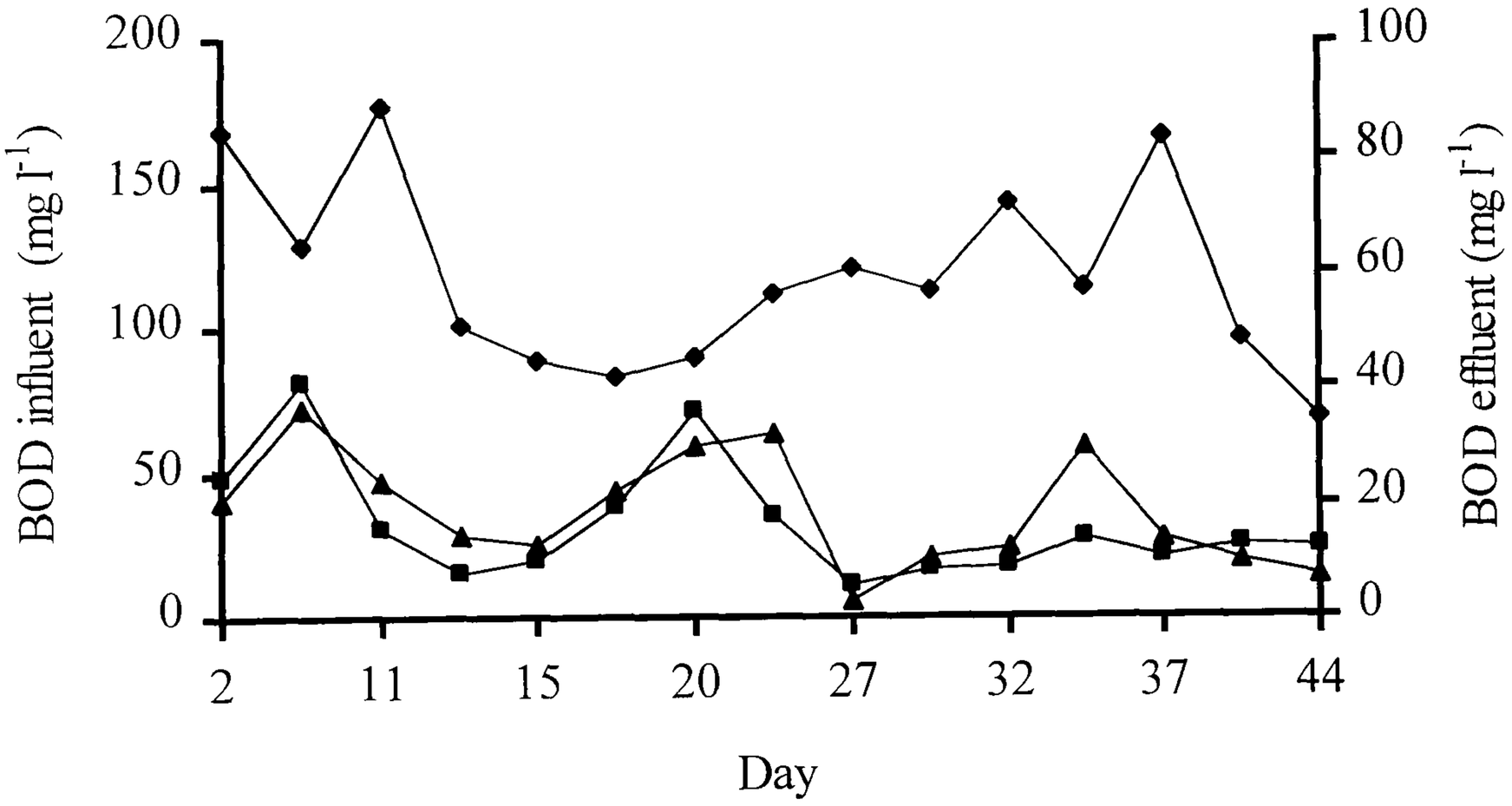

Figure 10.2: Influent (◆) and effleunt BOD (mg l^{-1}) in untreated (■) and 2,4 DNP treated (▲) activated sludge effluent (Chemical treatment began at day 14).

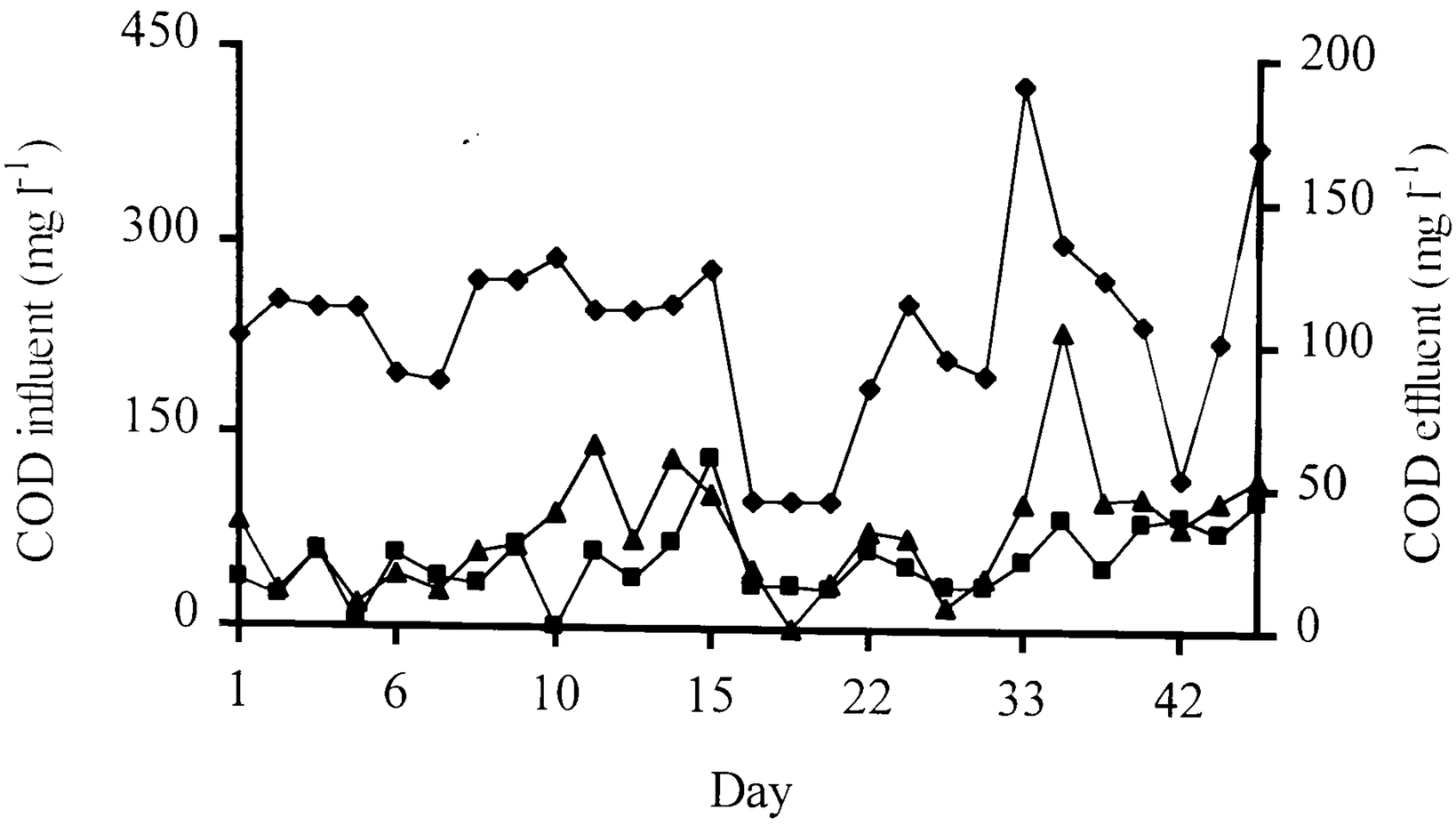

Figure 10.3: Influent (◆) and effleunt COD (mg l^{-1}) in untreated (■) and 2,4 DNP treated (▲) activated sludge effluent (Chemical treatment began at day 14).

After 5 weeks of chemical treatment, the BOD removal was not significantly different between the control (86.6 ± 9.1) and 2,4 DNP (84.6 ± 9.1) treated activated sludge (t = 1.15, P>0.05). Percentage removal figures of COD removal showed a small but statistically significant reduction in removal when treated with 2,4 DNP (t= 2.4, P<0.05) (Table 10.2).

Table 10.2: COD and BOD removal efficiencies (% ± SD) of untreated and 2,4 DNP treated activated sludge at pilot scale

	Control	2,4 DNP treated
BOD removal n=15	86.6 ± 9.1	84.6 ± 9.1
COD removal n=26	89.2 ± 6.7	85.7 ± 8.1

Nitrification was successfully achieved throughout the trial in both test and control channels (Table 10.3). The mean alkalinity during the trial period was 250.5 ± 16.4 mg l^{-1} as $CaCO_3$ and consequently enough to allow full nitrification.

Table 10.3: Nitrification in a pilot plant with untreated and 2,4 DNP treated activated sludge

		Control	2.4 DNP treated
NH_3–N removal (%)	pre treatment n=12	84.8 ± 3.2	74.5 ± 21.9
	post treatment n=16	85.5 ± 5.1	84.9 ± 5.6
NO_2 -N production (mg l^{-1})	post treatment n=16	0.5 ± 0.7	0.2 ± 0.2
NO_3-N production (mg l^{-1})	post treatment n=16	24.6 ± 5.0	26.1 ± 5.5

The mean influent ammonia concentration was 31.9 ± 6.7 mg l^{-1} and in the control effluent was 4.6 ± 1.1 mg l^{-1}, the 2,4 DNP treated effluent was 6.1 ± 4.5 mg l^{-1} (Figure 10.4).

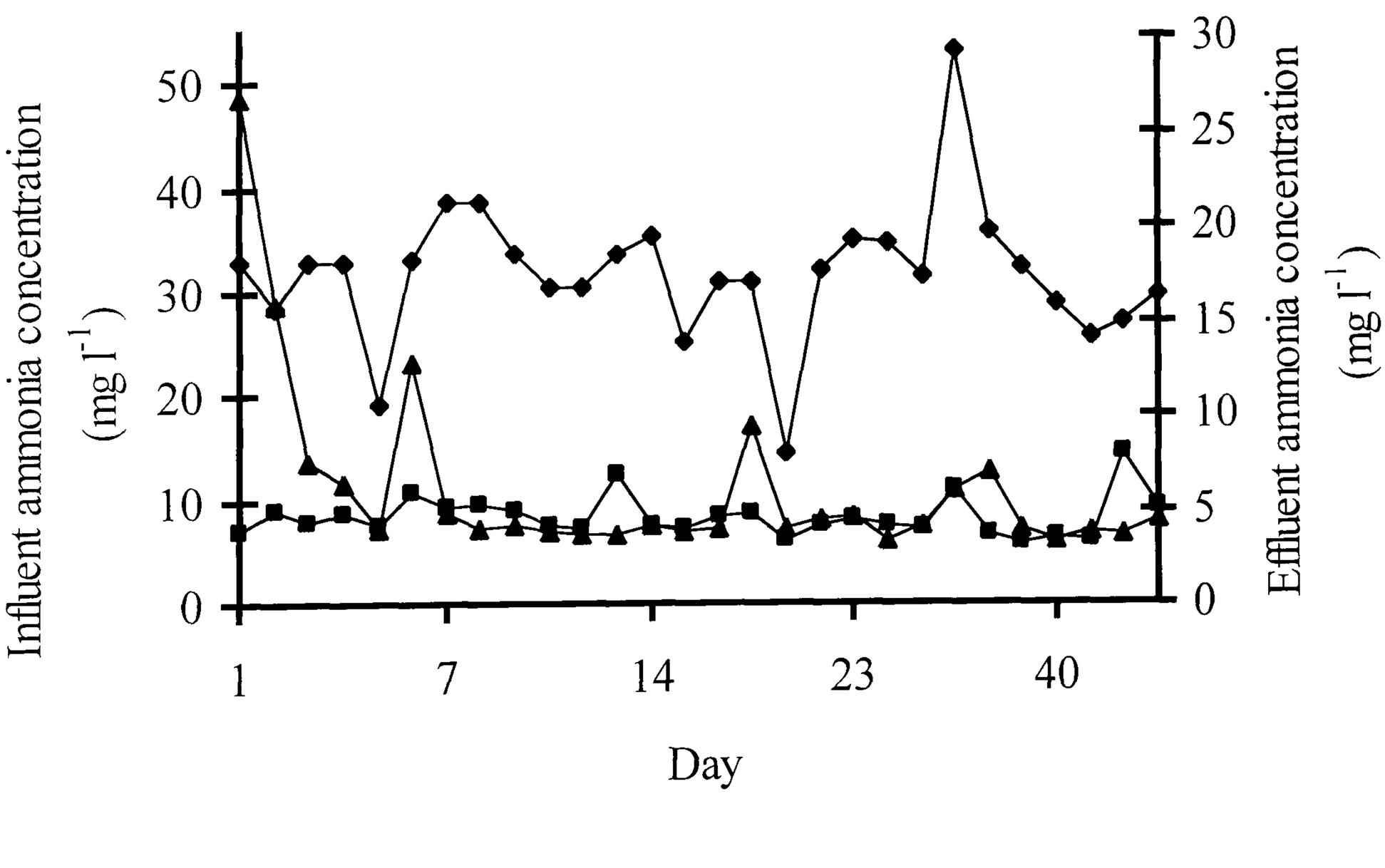

Figure 10.4: Influent (◆) and effleunt ammonia concentration (mg l^{-1}) in untreated (■) and 2,4 DNP treated (▲) activated sludge (Chemical treatment began at day 14).

Table 10.4: Mean (± SD) SVI and CST measurements in untreated and 2.4 DNP treated activated sludge

	Control	2,4 DNP treated
SVI (ml g^{-1}) n=15	264.4 ± 113.4	262.1 ± 99.3
CST (s) n= 6	5.5 ± 0.3	5.5 ± 0.3

The volatile fraction of the total solids indicates the active fraction. The control pilot unit had 93.3 ± 31.5 % volatile solids during the trial and the 2,4 DNP treated activated sludge 98.5 ± 18.7 % before treatment and 93.9 ± 18.6 % post treatment. Measurement of the volatile solids showed that addition of 2,4 DNP had no significant effect on the proportion of volatile solids.

Prior to chemical addition the oxygen uptake rates were not significantly different between the two units. After 2,4 DNP addition the oxygen uptake rate of the treated unit was significantly greater than the control (t= 3.96, P<0.05) (Table 10.5).

Table 10.5: Mean oxygen uptake rates for untreated and 2,4 DNP treated activated sludge (mg O_2 g MLSS $^{-1}$ h^{-1}) n = 4 ± SD

	Untreated	2,4 DNP treated
Pre treatment	0.43 ± 0.28	0.20 ± 0.07
Post treatment	0.33 ± 0.19	0.76 ± 0.07

The yield of biomass production based on BOD removal showed that the unit treated with 2.4 DNP was significantly lower after treatment; 1.08 ± 0.46 pre treatment and 0.77 ± 0.32 (t=2.16, P<0.05). Compared to the control the chemically treated activated sludge was significantly lower in yield (t=2.86, P<0.05) after treatment with 2.4 DNP

(Table 10.6, Figure 10.5). Two units were operated simultaneously in order to obtain a realistic comparison. However, the yields before chemical treatment showed that the pilot to be treated with water had a mean yield lower than that of the system to be chemically treated. The best comparison then is between the pretreatment and post treatment data of the single units in which the yield was significantly lower in the treated system and significantly higher in the untreated system ($t=1.83$, $P<0.05$).

Mean effluent suspended solids were not significantly different between treated and untreated pilot systems ($t=0.54$, $P<0.05$). The mean effluent suspended solids were 47.2 ± 38.9 mg l^{-1} in the control and 44.4 ± 19.4 mg l^{-1} in the 2,4 DNP treated activated sludge system. In both cases they were higher than expected for a full scale system due initially to enlarged numbers of filamentous organisms. When treated with 2,4 DNP the effluent suspended solids appeared to be less variable and a little lower than the control.

Table 10.6: Yield coefficients based on BOD removed (± SD) of untreated and 2,4 DNP treated activated sludge

	Untreated	2,4 DNP treated
Pre treatment n= 14	0.77 ± 0.35	1.08 ± 0.46
Post treatment n= 20	1.14 ± 0.74	0.77 ± 0.32

Microbiological analysis of activated sludge samples indicated that addition of the chemical had no significant effect on the species diversity index of the sludge; 16.0 in the untreated and 17.0 in the 2,4 DNP treated. There was no significant difference in the numbers of protozoa occurring in the two pilot systems ($t=0.26$ $P<0.05$) per ml. The total numbers of flocs present in the activated sludge was comparable between test and control (Table 10.7). The size of the flocs were considerably smaller in the 2.4 DNP treated activated sludge, the majority were small whereas in the untreated sludge the numbers of flocs were more evenly distributed between the 3 size categories. In

both pilot trials the flocs were predominantly firm and compact in structure. Treatment with 2,4 DNP progressively increased numbers of open flocs.

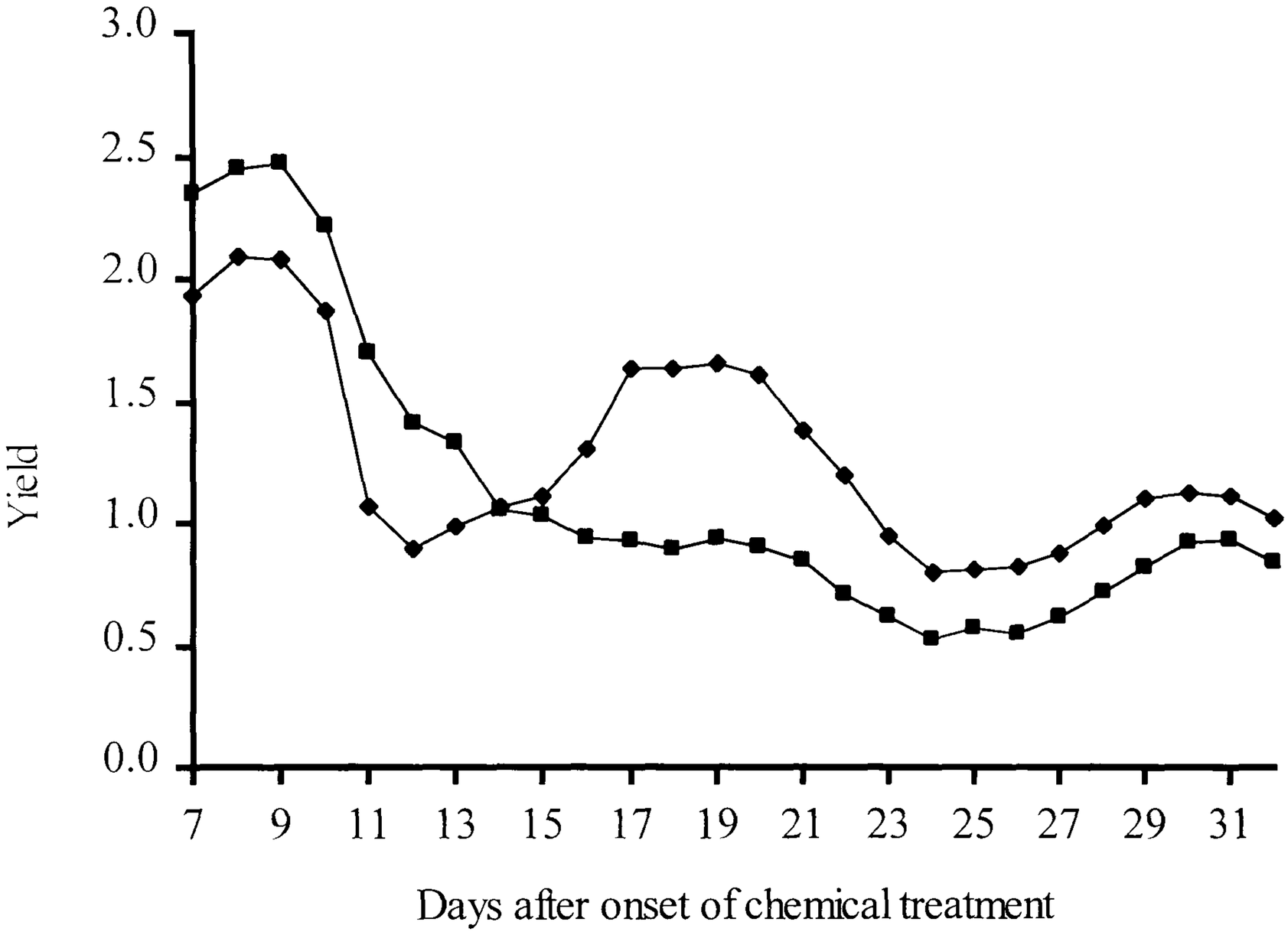

Figure 10.5: Moving average (7 d) of yield coefficients of (◆) untreated and (■) 2,4 DNP treated activated sludge

Table 10.7: Numbers and size of flocs per ml x 10^5 in untreated and 2,4 DNP treated activated sludge

Size of floc	Untreated	2,4 DNP treated
small	22	31
medium	22	15
large	11	5
Total	55	51

10.4 DISCUSSION

The pilot study showed that addition of 2,4 DNP to an activated sludge system did not affect the removal efficiencies in terms of COD, BOD and ammonia for the duration of the trial period. The ammonia removal was not significantly different between test and control. Tomlinson *et al.* (1966) reported that the concentration of 2,4 DNP required for 75 % inhibition of ammonia oxidation by activated sludge was 460 mg l^{-1} or 2.5 x 10^{-3} M. The concentration applied in the pilot was 2.7 mg l^{-1} in the influent, consequently, based on the data of Tomlinson *et al.*, little inhibition of ammonia oxidation would be expected. One mechanism of biodegradation of 2,4 DNP is oxidative elimination of the nitro group, and as such nitrate is a known breakdown product of 2,4 DNP (Simpson and Evans, 1953; Haghighi-Podeh and Bhattacharya, 1996). Hess *et al.* (1990) reported that 2,4 DNP biodegradation by an *Actinomycete* gave increased nitrate production, and that the biodegradation was enhanced by supplemental addition of glucose. In this study there was no significant difference between the nitrate production of the untreated and 2,4 DNP treated activated sludge suggesting that no significant biodegradation of the 2,4 DNP occurred in the trial. This indicated that uncoupling was successfully achieved.

Uncoupling in wastewater treatment results in changes such as increased oxygen consumption and a decreased net synthesis (Okey and Stensel, 1995). The activated sludge in the pilot plant exhibited a increase in oxygen consumption rate when treated with 2,4 DNP. Experiments carried out using a culture of *Klebsiella* treated with 2,4 DNP caused an almost constant increase in oxygen consumption rate. This was interpreted as being a result of a constant rate of energy dissipation by the chemical which is consistent with Mitchell's hypothesis of uncoupling (Neijssel,1977). Uncoupling by 2,4 DNP is supported by work using whole animals in which the increased oxygen consumption can be purely attributed to an increasing metabolic rate and not increased activity as the animals were kept immobile (Schultz and Cronin, 1997).

The CST is used to determine the dewaterability of sludge and is the time that filtrate takes to travel a fixed distance on filter paper (Chen *et al*.. 1996). The larger the value the poorer the filterability characteristics of the sludge. Since the CST measurements of both treated and untreated activated sludge were similar the results suggested that addition of the uncoupler would not affect the properties of the resulting reduced volume of waste sludge. That is, less sludge would be produced with the same dewatering and settling characteristics so that no processing changes would be necessary. A low SVI value indicates good settling properties; the mean SVI in both the test and control pilot unit were quite high due to the initial presence of filamentous organisms. Both were similar suggesting that chemical addition did not affect the settling properties of the activated sludge despite the formation of smaller size flocs. Yasui *et al*. (1996) carried out a pilot trial to investigate the reduction in excess sludge by a recirculation of sludge via ozonation; SVI figures of 200-300 ml g^{-1} were reported similar to those found in this study.

Removal of organic matter has been shown to be stimulated by increasing addition of 2,4 DNP (Rich and Yates, 1955) up to a concentration of 25 mg l^{-1}, but above this the rate was decreased. Conflictingly, Okey and Stensel (1993) reported that addition of 2,4 DCP (which has the same basic carbon skeleton as 2,4 DNP) reduced substrate uptake by a factor of 2 to 5. In this study removal of organic material in terms of BOD was not detrimentally affected by the addition of 2,4 DNP but no stimulation of removal was observed. COD removal was slightly lower when activated sludge was treated with 2,4 DNP; adding 2,4 DNP increased the COD of the system. As the 2,4 DNP was not degraded such an increase was expected.

Using a recirulation system via ozonation, Yasui *et al.* (1996) reported that most wastewaters could be treated with zero excess sludge production. They described that some build up of inorganic matter occurred which aided in settlement, producing lower SVI figures. In the pilot scale experiment carried out in this study, the percentage VSS did not significantly alter on addition of chemical. This suggested that the proportion of inorganics did not alter either. This may be beneficial, as the activated sludge

remaining in the aeration tanks would still have a high proportion of viable active biomass.

The aeration tank to be dosed with 2,4 DNP was stable at a greater MLSS than the channel to be the control. However after 7 d of chemical treatment, the yield of the treated sludge dropped to below that of the control and remained significantly lower throughout the experimental period. Previous smaller scale research (Chapter 8) reported a reduction in yield from 0.42 ± 0.09 in the control to 0.30 ± 0.05 when treated with 2,4 DNP. The reduction seen in this pilot study was greater than in the previous simulation; 0.66 ± 0.30 in the untreated to 0.41 ± 0.06 in the chemically treated, a lower and less variable yield. The yield obtained in the control pilot system was fractionally higher than the typical yield of the conventional activated sludge process; 0.6 (Metcalf and Eddy 1991). The yield reduction resulting from 2,4 DNP addition was comparable to that obtained by other process alterations (Chapter 5), however most of these other systems involve separate treatment stages. This pilot study demonstrated the simplicity of using chemical uncouplers; the chemical was supplied directly to the aeration chamber and after a few days lag, a sustainable reduction in yield was observed.

10.5 CONCLUSIONS

- An activated sludge pilot plant was successfully run with the addition of the uncoupler 2,4 DNP without causing cell kill or performance deterioration.
- The yield of MLSS was significantly reduced from 1.14 in the control to 0.77 in the 2,4 DNP treated activated sludge.
- The yield reduction was achieved without detriment to process performance in terms of BOD and ammonia removal.
- COD removal was slightly reduced with treatment of 2,4 DNP.
- Less nitrite was produced in the 2,4 DNP treated unit suggesting that the extent of nitrification in the treated unit was greater than that of the control.
- There was no significant difference in the amount of nitrate produced between the chemically treated and control units; indicating that no significant amount of biodegradation of the chemical occurred.
- The species diversity index of the 2,4 DNP treated sludge was one unit greater than that of the control suggesting that the activated sludge was marginally more diverse. However, there was no significant difference in the total numbers of protozoa occurring per unit volume of activated sludge.
- After chemical treatment the tendency was for greater numbers of smaller flocs to occur; which may be detrimental to final effluent quality if pin point floc size is reached and carry over into the final effluent occurs.

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Chapter 11: Discussion

Chapter 11

Discussion

11.1 OVERVIEW

The need for reduction of waste biomass at source in the activated sludge process has become increasingly important. Attempts to optimise process control and use of alternative configurations have been investigated with a view to lowering biomass production. Although several adaptations have resulted in lowered biomass yields and consequently reduced waste, implementation required capital works for extra chambers or increased costs often in the form of energetic inputs for oxygen supply.

Preliminary investigations into the use of chemical uncouplers in both pure culture and activated sludge suggested that such a system had potential to reduce biomass yield (Low and Chase, 1998). The current research identified several chemicals with the potential to interact with microbial metabolism to lower biomass production; from known uncouplers to TCA cycle inhibitors and antibiotics. Biomass reduction in the activated sludge process will only be successful if it is not at the expense of process performance.

Many chemicals were tested to identify feasibility for use in minimisation of sludge production. Ideally, the requirements for a yield reduction chemical would be an uncoupling action (to allow continuation of catabolic paths and sewage pollutant degradation); non-toxic to mammalian cells, preferably specific to bacteria and internalised on reaction with the microorganisms; and in case of excess being washed out in the effluent have the capability for removal before effluent streams reach the receiving water course.

Laboratory tests identified that selection of the correct chemical and appropriate concentration can result in yield reduction without significant detriment to COD, BOD

or ammonia removal. Investigations with a glucose substrate resulted in chemically treated activated sludge samples obtaining a COD substrate degradation rate either comparable to or enhanced compared to the control. The greatest stimulation of COD removal was obtained by 2,4 DNP and rotenone. This trend was repeated in activated sludge simulations using a settled sewage substrate where percentage removal figures of both COD and BOD were not statistically different to the controls. Previous research is conflicting on this issue: Rich and Yates (1955) noted a stimulation of organic matter removal in activated sludge treated with 2,4 DNP, and Low and Chase (1998) reported an increase in the specific rate of substrate degradation of a *Pseudomonas* culture treated with 4 NP. However, Okey and Stensel (1993) found a decrease in the rate of phenol and glucose uptake in activated sludge treated with 2,4 DCP and other uncouplers.

Literature suggested that most chemicals have a detrimental impact on nitrification ability of activated sludge (Richardson, 1985); the inhibition tests carried out reflected the amount of inhibition to be both chemical type and concentration dependant. 2,4 dinitrophenol caused a negligible 8 % inhibition, whereas the more powerful uncoupler 4 NP resulted in upto 90 % inhibition of both ammonia removed and nitrate produced. At pilot scale the nitrification ability of the 2,4 DNP treated sludge was not significantly different (in terms of ammonia removal, nitrite and nitrate production) to the control.

Transient addition of nitrophenol uncouplers and chlorophenols showed an increase in oxygen consumption in activated sludge (Clowes *et al.,* 1950; Okey and Stensel, 1993; Okey and Stensel, 1995; Rich and Yates, 1955). Therien *et al.* (1984) demonstrated that below a critical dose (of aliphatic alcohol addition to activated sludge) the specific oxygen uptake rate rose rapidly, but above this concentration the oxygen consumption decreased with chemical concentration. Such observation suggested that the concentration was critical to achieve uncoupling. Different concentrations of chemicals exhibited different oxygen uptake rates highlighting the need for identification of the correct dose to achieve successful uncoupling.

The increased oxygen uptake rates suggested uncoupling was occurring, and so theoretically, biomass reduction was underway. Batch operated tests of the chemicals promoting the greatest oxygen uptake rates (2,4 DNP, rotenone, trypan blue) resulted in reduced MLSS accumulation. However, in an activated sludge simulation rotenone did not maintain this yield reduction; a reduction in MLSS occurred but there was no significant effect on yield. 2,4 DNP and 4 NP both successfully reduced yield of the activated sludge process compared to the control in bench scale simulations. The scale-up of small respirometry tests was vital, as some chemicals proved inadequate at biomass reduction on a larger scale despite promising initial tests.

The increased oxygen uptake rate demonstrated by uncoupler addition indicated the potential for greater process efficiency; as loading rates may possibly be increased without detriment to removal rates with the increased catabolic activity of the microorganisms. Thus any increase in oxygen supply to support the system may be balanced against the increased loading capability of an activated sludge plant.

For the implementation of uncouplers in full scale works any washout of the chemicals in the effluent needs to be controlled and removed. The nature of uncouplers involves action from within the microorganisms. Uncouplers generally possess a weak acid unit (amino or hydroxyl group), a hydrophobic aromatic moiety and electronegative groups such as nitro or halogen substituents (Schultz and Cronin, 1997). Lipophilicity allows passive diffusion across biological membranes at physiological pH (Riveranevares *et al.*, 1995). The chemical will then be retained in the microorganisms. All the chemical should be taken up providing overdosing does not occur.

In the event of chemical passage through to the effluent several solutions are available for removal. Both 2,4 DNP and 4 NP are coloured compounds and can be degraded photochemically. 2,4 DNP can be degraded by sunlight alone. A solution with a concentration of 200 mg l^{-1} can be totally degraded within 2 months (Verschueren, 1996). 2,4 dinitrophenol is toxic to microorganisms at concentrations of 110 mg l^{-1} (EC_{50}=110 mg l^{-1}) and the LD_{50} for 2,4 DNP to mice is 45 mg kg^{-1} (that is the dose that

is lethal to 50% of the organisms tested). The solution that was used in the pilot trial was 500 mg l^{-1}, so sufficient sunlight exposure has the potential to reduce the chemical levels to below those considered toxic. Toxicity would be reduced further by dilution into the receiving water.

Current research has reported the successful elimination of phenols from wastewater by photochemical methods including ultra violet light (UV), hydrogen peroxide (H_2O_2) and heterogeneous photocatalysts (Chen *et al.,* 1997; Kang *et al.,* 1998). Ozone has been demonstrated to achieve complete and rapid oxidation of substituted phenol wastes (Mokrini *et al.,* 1997). Any plant already using such techniques or ozone treatment can easily be updated to use biomass reduction with uncouplers and have an existing method of cleaning effluent if required. Other methods of removal exist for 2,4 DNP including absorbance onto bagasse fly ash (a waste product of the sugar industry) (Srivastava *et al.,* 1995) or a carbonaceous adsorbent derived from fertiliser waste (Sirvastava *et al.,* 1997).

Uncouplers are advantageous as they are universal and will interfere with the metabolism of any cell. More direct interactions with microorganisms are becoming possible by genetic manipulation (McClure *et al.*, 1991), but as yet the long term implications of such technology are unknown and it is environmentally unacceptable to release genetically modified organisms into the environment (Hoekstra, 1990). Potential application of genetic engineering in wastewater treatment has inherent difficulty in tackling sufficient microorganism species in the mixed culture to decrease the biomass production.

The risk of microorganisms developing resistance to the addition of any chemical is always present. Many cases of antibiotic resistance are documented and some specific strains of bacteria have been found to degrade uncoupling chemicals (Harris *et al.*, 1995). However to stimulate this chemical degradation, specific conditions are required such as the presence of a specific substrate like glucose, a specific concentration of chemical or the presence of an additional chemical (Gage and

Neidhardt, 1993) or by mutation (Lewis *et al*, 1994). Activated sludge has a huge diversity of species and a continual turnover of new species lessening the likelihood of chemical resistance building up.

Careful process control has the potential to reduce biomass production. Lowering the F:M ratio by increasing the amount of MLSS in the aeration basin or reducing the influent load can prevent the accumulation of biomass. However the capacity of the clarifiers to deal with the higher solids loads and fluctuating flows may prevent such operation on a continual basis. Increasing sludge age (by ceasing wastage or increasing MLSS) can prevent sludge needing disposal by promoting both protozoa growth and cell decay, but results in an increased demand for oxygenation (Maclennan, 1994). It may be argued that in the simulations carried out here, such operational controls were in action, however an untreated and test system were run concurrently in all cases and if physical controls were in operation then they had identical influence in both systems. The yield reduction seen was attributed to the chemical effect and not other attributes such as sludge age (Table 11.1).

Table 11.1: Comparison of sludge age and F:M ratio of all activated sludge simulations

		Sludge age		F:M ratio	
Chemical	Dose mg g^{-1} d^{-1}	Treated	control	Treated	control
Rotenone	0.29	12.9 ± 1.8	11.4 ± 1.5	0.61 ± 0.12	0.60 ± 0.07
4 NP	0.02	12.4 ± 4.0	10.8 ± 4.8	0.27 ± 0.12	0.24 ± 0.09
2,4 DNP	7.00	1.51 ± 0.18	1.54 ± 0.24	0.19 ± 0.08	0.18 ± 0.09
2,4 DNP (pilot)	3.20	6.7 ± 0.26	6.1 ± 1.3	0.25 ± 0.26	0.21 ± 0.09

The addition of chemical (rotenone, 2,4 DNP or 4 NP) had little effect on the diversity of the protozoa species present (Table 11.2). This suggested that chemical presence did not affect the numbers or type of protozoa occurring. This was reflected in the

process performance, as chemical addition did not reduce the BOD or COD removal capability.

Floc sizes were observed to be smaller but occurring in similar numbers per unit volume. Formation of small flocs may be detrimental to the quality of the final effluent. When the flocs are of such a small or 'pinpoint' size they often fail to settle out in clarifiers and carry out with the effluent thus increasing the suspended solids in the effluent (Tillman, 1996). The formation of small flocs in the pilot trial with 2,4 DNP did not affect the settleability in terms of the SVI and CST which were comparable in both test and control (Chapter 10).

Table 11.2: Effect of chemical addition on activated sludge species diversity

Chemical treatment	Control	Treated
2,4 DNP[1]	16.0	17.0
4 NP	16.9	17.7
Rotenone	14.5	16.0

([1] 2.4 DNP at pilot scale, others at 3 l simulation)

It has already been identified that selection of an optimal concentration of chemical is important to achieve biomass reduction without causing cell death. Previous research is conflicting with regard to the type of dose measurement that is critical for maintaining this balance. Low and Chase (1996) stated that it was the absolute concentration not the ratio of 4 NP to biomass that was significant in *Pseudomonas* cultures. However, Okey and Stensel (1993) stated a specific range of mass of uncoupler to mass of MLVSS ratios between which the effect of the uncoupler was not toxic.

The doses used in these experiments were initially absolute in the respirometric tests, however as the tests were scaled up this became difficult to maintain. In the continuous simulations where an influent stream of both chemical and wastewater

caused a subsequent effluent, it was not possible to obtain the absolute concentration of chemical in the aeration vessel at any one time. Since the compounds were water soluble, chemical that was not taken up by the biomass could be carried over to the effluent. Further research should address the issue of determination of optimal chemical concentrations and subsequently identify the most suitable dosing regime (absolute concentration or mass to mass ratio). Although absolute concentrations are repeatable and simple in closed situations were the volume and solids content of the sample under test do not alter, this is not practical on a large scale involving continuous treatment. From the doses actually applied in the continuous experiments dose rates of between 0.02 and 7.0 mg g $MLSS^{-1}$ d^{-1} were applied (Table 11.1). Since the effluent was coloured and probably overdosed as a result of chemical addition in the small scale simulation with 2,4 DNP the dose was decreased from 7.0 to 3.2 mg g $MLSS^{-1}$ d^{-1} in the pilot scale trial. The concentrations and supply rates utilised in the simulations were carefully selected; however it is likely that these could be further optimised to obtain increased biomass reduction and keep costs to a minimum.

The fears that the promising effects seen in the respirometric testing and experiments carried out in pure culture (Low and Chase, 1996 and 1998) may not be obtainable at large scale have been diminished by the successful pilot trial. The biomass yield was lowered in a larger scale system with natural conditions and settled sewage feed. Although larger scale experiments (possibly the installation of chemical treatment at an existing sewage treatment works as a first reference site) may be required to reinforce the capability of this treatment; the results presented indicate the sustainability of chemical uncoupling in the activated sludge process.

11.2 SLUDGE PRODUCTION

Legislation has forced the issue of reducing waste sludge at source. As restrictions on how and where sludge can be disposed tighten the cost of disposal rises (Hall, 1996). Considerable beneficial cost implications are likely with any system capable of reducing waste sludge. The use of chemical inhibitors does not require any capital works and the only additional operating costs are in aeration and chemical supply.

Using standard calculations (Metcalf and Eddy, 1991) the amount of waste sludge produced can be determined based on the operating parameters of the two pilot streams:

$$P_x = Y_{obs}Q(S_0 - S) \times (10^3)^{-1} \qquad (11.1)$$

Where: P_x = net waste activated sludge produced each day in terms of MLSS (kg d^{-1})

Y_{obs} = observed yield

Q = influent flow rate (m^3 d^{-1})

S_0 = average influent BOD (g m^3)

S = average effluent BOD (g m^3)

Table 11.3: Pilot trial parameters

Parameter	Control system	2,4 DNP treated system
Y_{obs}	1.14	0.77
Q	0.95	0.95
S_0	150	150
S	16	18

Using the values from the pilot trial (Table 11.3):

$$P_{xcontrol} = 1.14.\ 0.95(150\text{-}16) \times (10^3)^{-1}$$

$$= 0.145 \text{ kg } d^{-1} = 52.9 \text{ kg per year} \qquad (11.2)$$

$$P_{x2,4\ DNP\ treated} = 0.77.\ 0.95(150\text{-}18) \times (10^3)^{-1}$$

$$= 0.097 \text{ kg } d^{-1} = 35.4 \text{ kg per year} \qquad (11.3)$$

From equations 11.2 and 11.3 a saving of some 17.5 kg waste activated sludge per year in the pilot plant alone (330 l, treating 0.95 m^3 d^{-1}) was saved. If these figures are extrapolated to consider a full scale works treating 35 000 m^3 d^{-1} then production of

654 t y^{-1} of sludge per year is avoided (equations 11.4 and 11.5). The benefits of this are in the savings in digestion and disposal.

$$P_{xcontrol} = 1.14.\ 35000(150\text{-}16)\ x\ (10^3)^{-1}$$

$$= 5347\ kg\ d^{-1} \qquad = 1952\ t\ y^{-1} \qquad (11.4)$$

$$P_{x2,4\ DNP\ treated} = 0.77.\ 35000(150\text{-}18)\ x\ (10^3)^{-1}$$

$$= 3557\ kg\ d^{-1} \qquad = 1298\ t\ y^{-1} \qquad (11.5)$$

11.3 COST ANALYSIS

The following sets out a cost analysis of using 2,4 DNP as a biomass reduction tool based on the experimental data obtained from the pilot trial extrapolated to a plant treating 35000 $m^3\ d^{-1}$. Several assumptions are used;

1. The cost of 2,4 DNP is 10% of the price of small laboratory quantities (£30.70 per kg) for bulk purchase - £3 per kg.
2. Addition of 2,4 DNP causes a theoretical increased oxygen requirement of 1.4 times compared to the control (see Appendix 3 for calculations).
3. The average dry weather flow is the same and is 35 000 $m^3\ d^{-1}$, this is treated in a 5800 m^3 working volume aeration basin with a MLSS of 2500 mg l^{-1} and hydraulic retention time of 4 h.
4. There is no requirement for new aeration facilities just an increased electrical power demand.
5. The only additional costs are that of the power and the chemical; no civil, mechanical or instrumentation costs are incurred.
6. The aeration system in place is 3 simplex aerators each using 10 kW of power, which is available at 6.6 p per hour for 14 h of the day and 2.6 p for the remaining 10 h.
7. The cost of waste sludge is £20 per tonne for digestion and £115 per tonne for disposal (1994 figures obtained from North West Water).

11.3.1: COST OF CHEMICAL

The rate of chemical addition to the pilot plant was 5.2 l d^{-1} of a 0.5 g l^{-1} solution which was based on addition to 2500 mg l^{-1} MLSS. This equated to a dose of 3.15×10^{-6} g mg^{-1} d^{-1}.

Chemical required daily $= V \times X \times CD$

$=5800000 \times 2500 \times (3.15 \times 10^{-6})$

$= 45.6$ kg d^{-1}

$=16, 644$ kg y^{-1} (11.6)

Where V = volume of aeration basin (l)

X = MLSS of aeration basin (mg l^{-1})

CD = chemical dose required daily (g mg^{-1} d^{-1})

Cost of chemical per year = 3 x 16, 644

=£49, 932 (11.7)

11.3.2: ELECTRICAL POWER COST

Cost for 3 aerators per year (total 30 kW) = 30 x 5110 h @ 6.6 p = £10,117

30 x 3650 h @ 2.6 p = £2,847

Total cost p. a. = £12,149 (11.8)

If addition of 2,4 DNP increases consumption by 1.4 then:

Cost of power for chemically treated sludge = 1.4 x 12,149 = £18,149 (11.9)

11.3.3: SAVINGS

Table 11.4: Cost comparison of chemically treated and untreated activated sludge

Cost per year	2,4 DNP treated	untreated
Cost of chemical (£)	49,932	-
Cost of electrical power (£)	18,149	12,964
Cost of treatment and disposal of sludge (£)	175,297	263,452
Total (£)	243,378	276,416

The total saved per year through chemical treatment would be £33,038 despite the increased power demand and chemical cost.

11.3.5. COST SENSITIVITY ANALYSIS

The saving possible is determined by the cost of the chemical to the treatment works. The assumed price of £3 per kg was based on information form the chemical supplier for bulk purchase. The current price for small laboratory scale analytical grade quantities is £30 per kg. The cost of treatment is lower than an untreated system up to a cost of £5 per kg (Table 11.5). Obviously the cheaper the price of the 2,4 DNP the greater the saving that can be made; however the key issue is that this type of chemical treatment would not increase the operating costs of a treatment works.

Table 11.5: Sensitivity analysis of chemical cost compared to an untreated system.

Cost of chemical (£/kg)	untreated	4	5	8
Cost per year (1510 kg) (£)	0	66,567	83,220	133,152
Total price of chemical, power and disposal (£)	276,416	260,022	276,666	326,598

These savings have the potential to be further enhanced in the future. 2,4 dinitrophenol can be found in the wastewaters of several industries such as the pharmaceutical and chemical production. If these industrial waste streams requiring treatment can be used as the source of uncoupler two problems may be solved in one stage – the treatment of the waste and the reduction of the biomass. If any chemical passing out in the effluent can be adsorbed (for example onto bagasse fly ash (Srivastava *et al.*, 1995 and 1997) the scope for chemical recovery and reuse arises which could reduce significantly the amount to be purchased.

There are several benefits of chemical uncoupling in the activated sludge process. The process can be easily retrofitted to any existing wastewater treatment works with limited increased operating expenditure. The reduced volume of sludge produced results in financial savings and reduces environmental pressures, as less sludge requires treatment and disposal.

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Chapter 12: Conclusions

Chapter 12
Conclusions

1. A total of 12 chemicals were identified with the potential to reduce biomass accumulation; they were in 3 groups: the uncouplers (2,4 DNP, 4 NP, rotenone, dicoumarol, quinacrine, chlorpromazine), antibiotics (erythromycin, vancomycin, oligomycin, antimycin) and TCA cycle inhibitors (trypan blue, congo red).

2. The effect of the 12 chemicals on oxygen uptake rate varied. In general the uncouplers caused an increase in the oxygen uptake rate and the antibiotics a decrease. Both effects suggested that a reduction in biomass was possible.

3. In laboratory tests, the COD removal rate of chemical inhibitor treated activated sludge was comparable to untreated activated sludge samples. Rotenone and 2,4 DNP both stimulated an increase in the COD removal rate. Dicoumarol was the only chemical to increase the COD concentration.

4. The effect of inhibitor addition on nitrification ability of activated sludge was varied. Inhibition of both ammonia removal and nitrate production of up to 80 % occurred with 4 NP. The least amount of inhibition of both ammonia removal and nitrate production occurred with 2,4 DNP (8 % and 4 % respectively).

5. The mean rate of ammonia removal (mg mg^{-1} h^{-1}) was more than halved by the addition of 4 NP, trypan blue, erythromycin and vancomycin compared to the untreated activated sludge rate of 2.4 mg mg^{-1} h^{-1}. Rotenone and 2,4 DNP had less effect reducing the rate to 1.7 and 2.1 mg mg^{-1} h^{-1} respectively.

6. Batch-fed tests with trypan blue, rotenone and 2,4 DNP resulted in reduced MLSS accumulation in treated activated sludge (52, 44 and 56 % reduction respectively) compared to the control (40 %). The extent of reduction was greater when

chemical was regularly added with the settled sewage feed rather than one initial dose (up to 80% reduction in MLSS).

7. In continuous laboratory scale activated sludge simulations rotenone, 2,4 DNP and 4 NP did not have a detrimental effect on process performance in terms of COD and BOD removal.

8. Both 2,4 DNP and 4 NP reduced the yield of activated sludge in continuous laboratory scale simulations. 2,4 dinitrophenol reduced the yield to 0.30 compared to the control value of 0.42. 4 nitrophenol reduced the yield to 0.19 compared to the control yield of 0.44. Rotenone had no significant effect on yield.

9. Yield reduction was successfully and sustainably achieved at pilot scale using 2,4 DNP. The control had a yield of 1.14 and the 2,4 DNP treated activated sludge 0.77. The process performance of the chemically treated activated sludge was not affected in terms of BOD, COD, ammonia removal, nitrate and nitrite production, SVI or CST.

10. Monitoring of the species diversity of activated sludge treated with rotenone, 4 NP or 2,4 DNP showed that chemical addition did not significantly alter the diversity of the protozoa occurring. A slight increase in diversity was observed, all the sludge samples appeared healthy which was reflected in the process performance.

11. Addition of 2,4 DNP and 4 NP caused a reduction in the size of flocs in the activated sludge, which was greater with 4 NP. This may be detrimental if pinpoint size flocs occur.

12. Addition of chemical inhibitors has the potential as a simple retrofit method for reducing biomass yield in the activated sludge process.

13. The addition of chemical inhibitors reduced MLSS accumulation in terms of Y_{BOD} at several scales of treatment without detriment to process efficiency.

14. Addition of a chemical uncoupler (2,4 DNP) was a cost effective method of reducing waste sludge production. Despite an increased oxygen demand savings of £33,000 were possible at a plant treating 35,000 m^3 d^{-1}, due to the reduced amount of sludge requiring treatment and disposal.

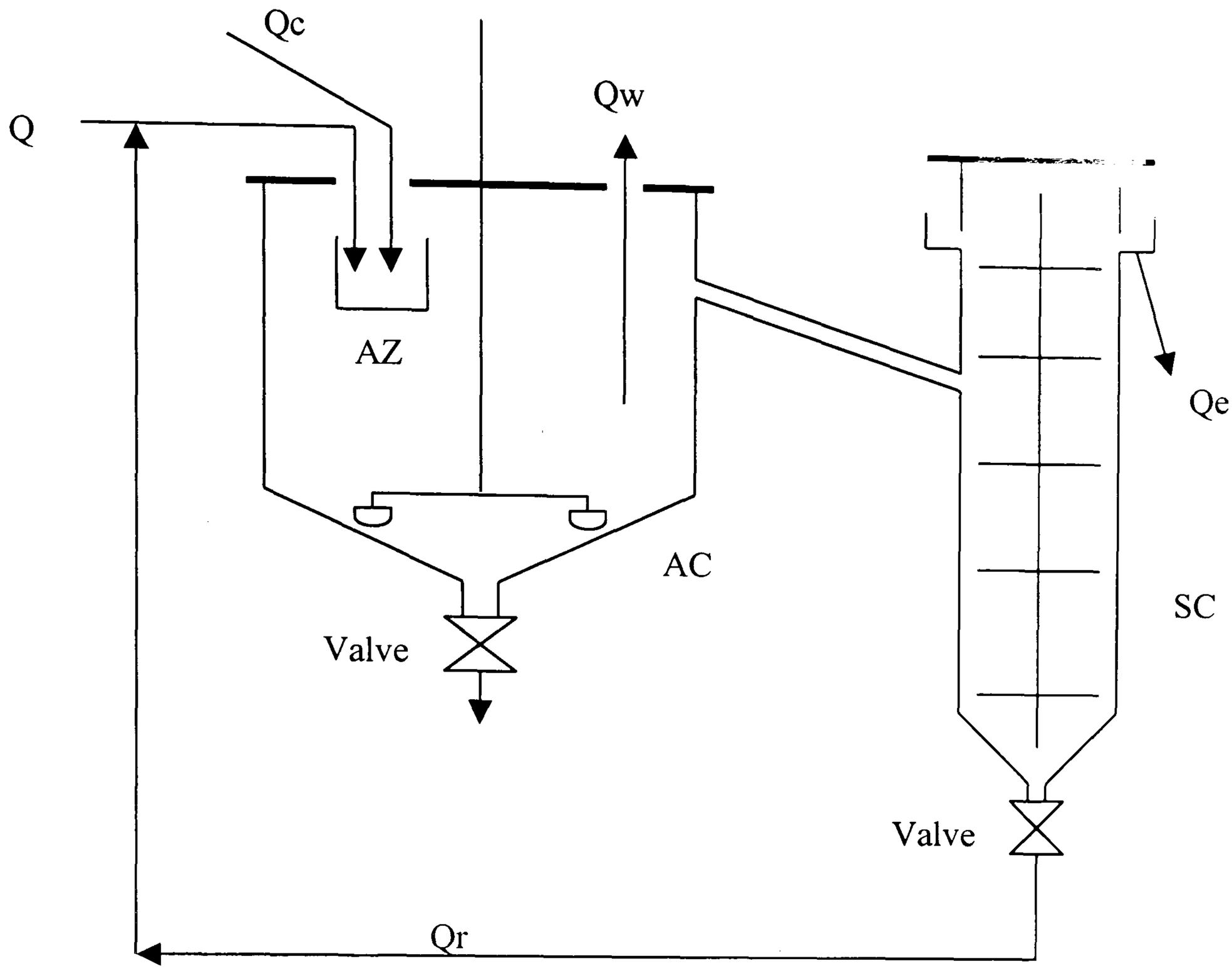
Appendices

1. Activated sludge pilot plant
2. The effect of chemical inhibitors on activated sludge floc structure
3. Theoretical oxygen demand

APPENDIX 1 :

ACTIVATED SLUDGE PILOT PLANT

Plate A1.1: Pilot plant at Cranfield University Sewage Treatment Works. a – aeration tank, s – settlement column, p- housing for all associated pumps



valve

One of four aerators per tank

AC - aeration chamber
SC - settling column containing scraper
AZ - anoxic zone
Q - influent flow
Qc - chemical flow
Qr - return sludge flow
Qe - effluent flow
Qw - waste sludge flow

Figure A1.1: Schematic of pilot plant

APPENDIX 2 :

THE EFFECT OF CHEMICAL INHIBITORS ON ACTIVATED SLUDGE FLOC STRUCTURE

A: PILOT SCALE TRIAL

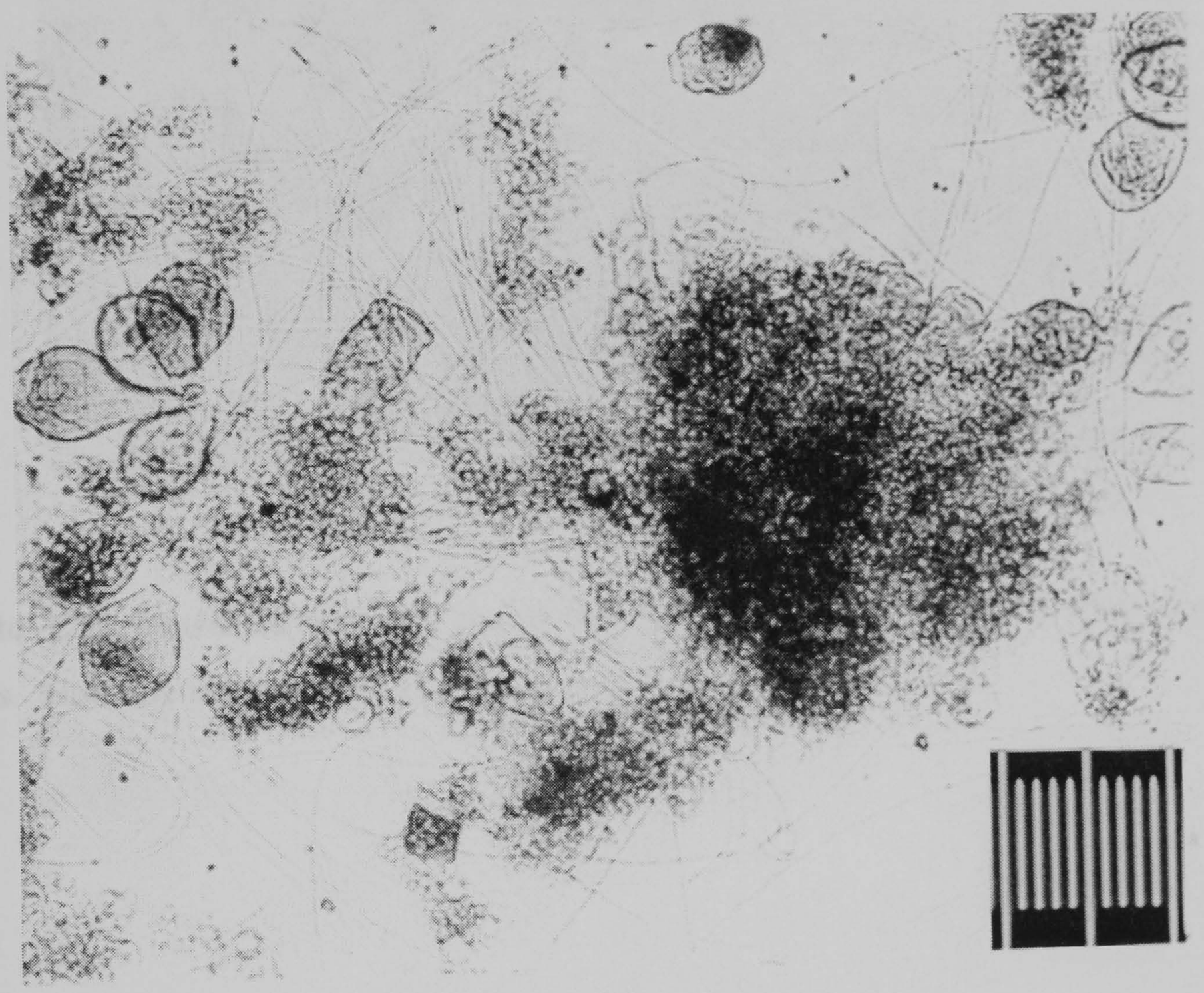


Plate A2.1: Untreated activated sludge from pilot plant. Scale – 0.5 mm between large bars. Note the large floc size and the large amount of filamentous strands visible before addition of the anoxic zone.

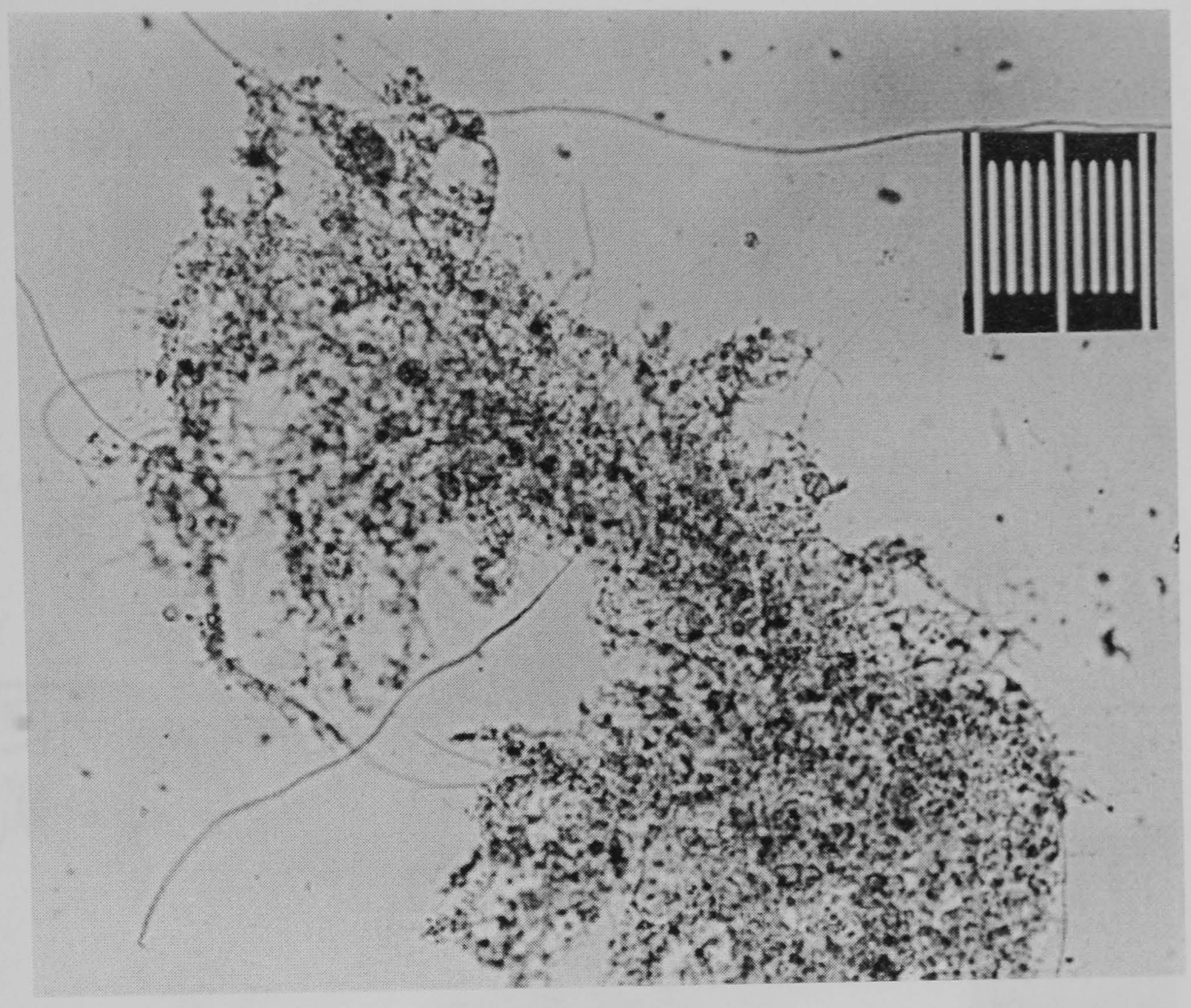


Plate A2.2: Untreated activated sludge from pilot plant. Scale – 0.5 mm between large bars. Note the large floc size.

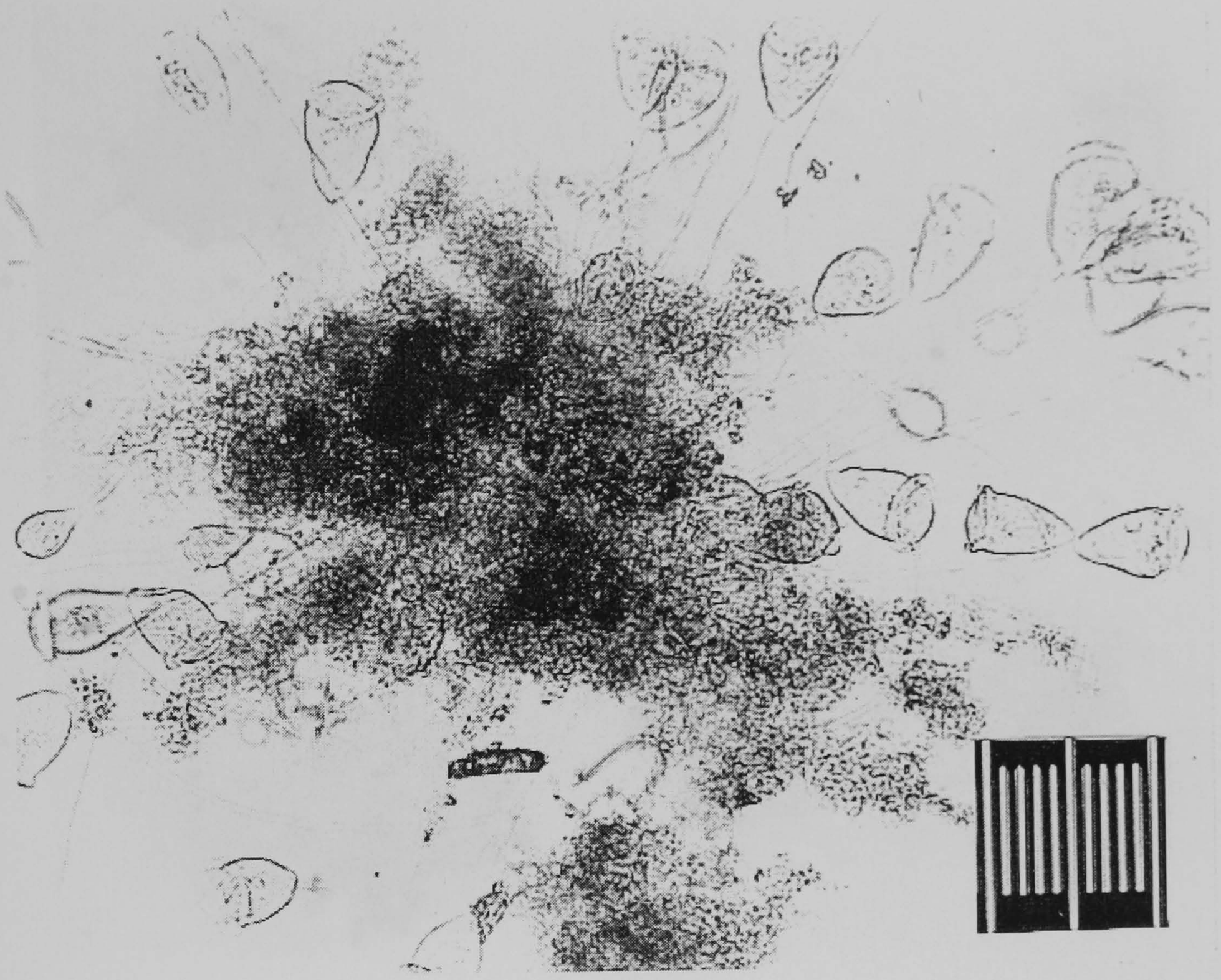


Plate A2.3: Untreated activated sludge from pilot plant. Scale – 0.5 mm between large bars. Note the large floc size and abundance of ciliates.

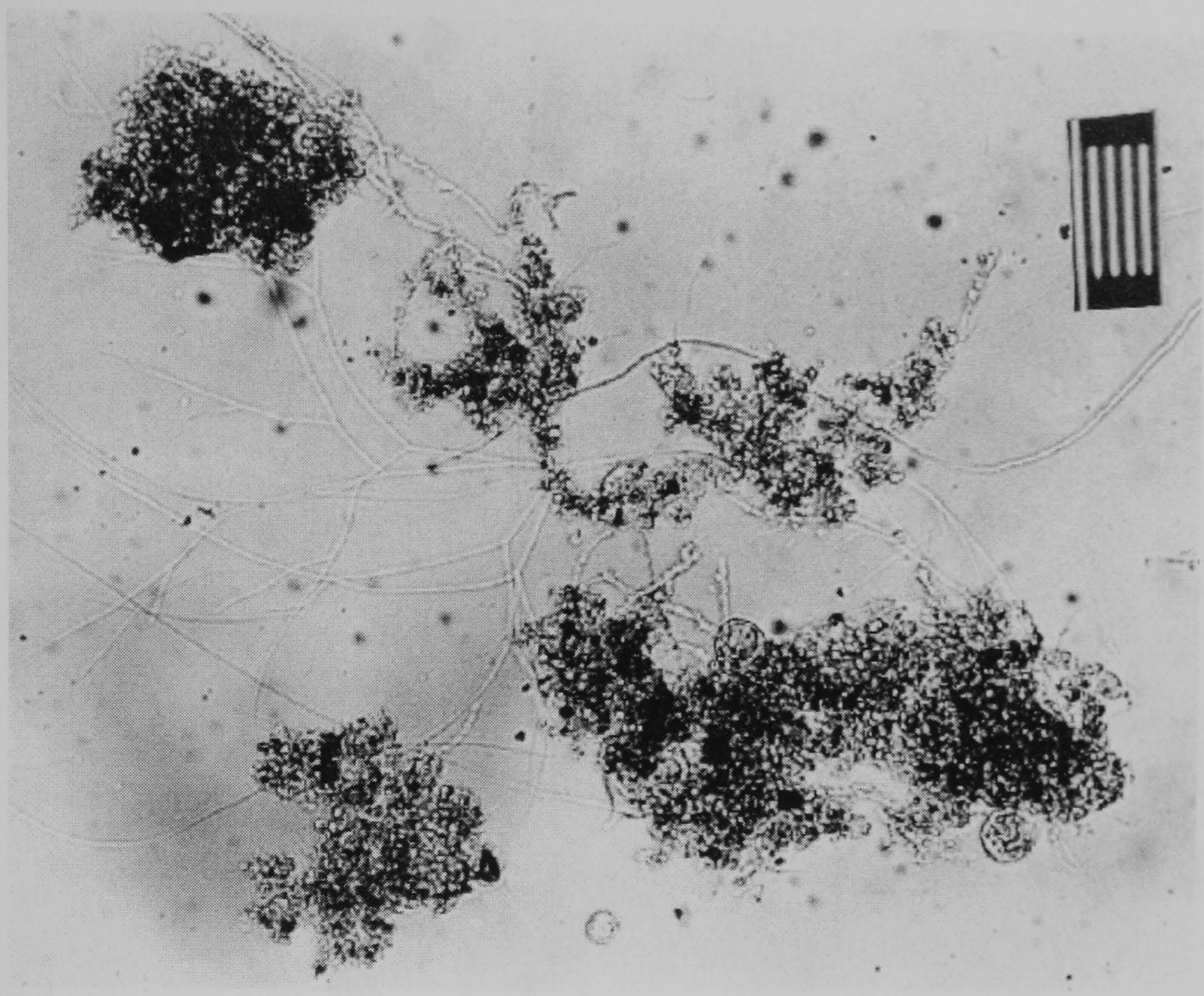


Plate A2.4: Activated sludge treated with 2,4 DNP at pilot scale after 25 d of chemical dosing. Each scale line 0.01 mm apart. Note that the flocs are still compact in nature but small in size.

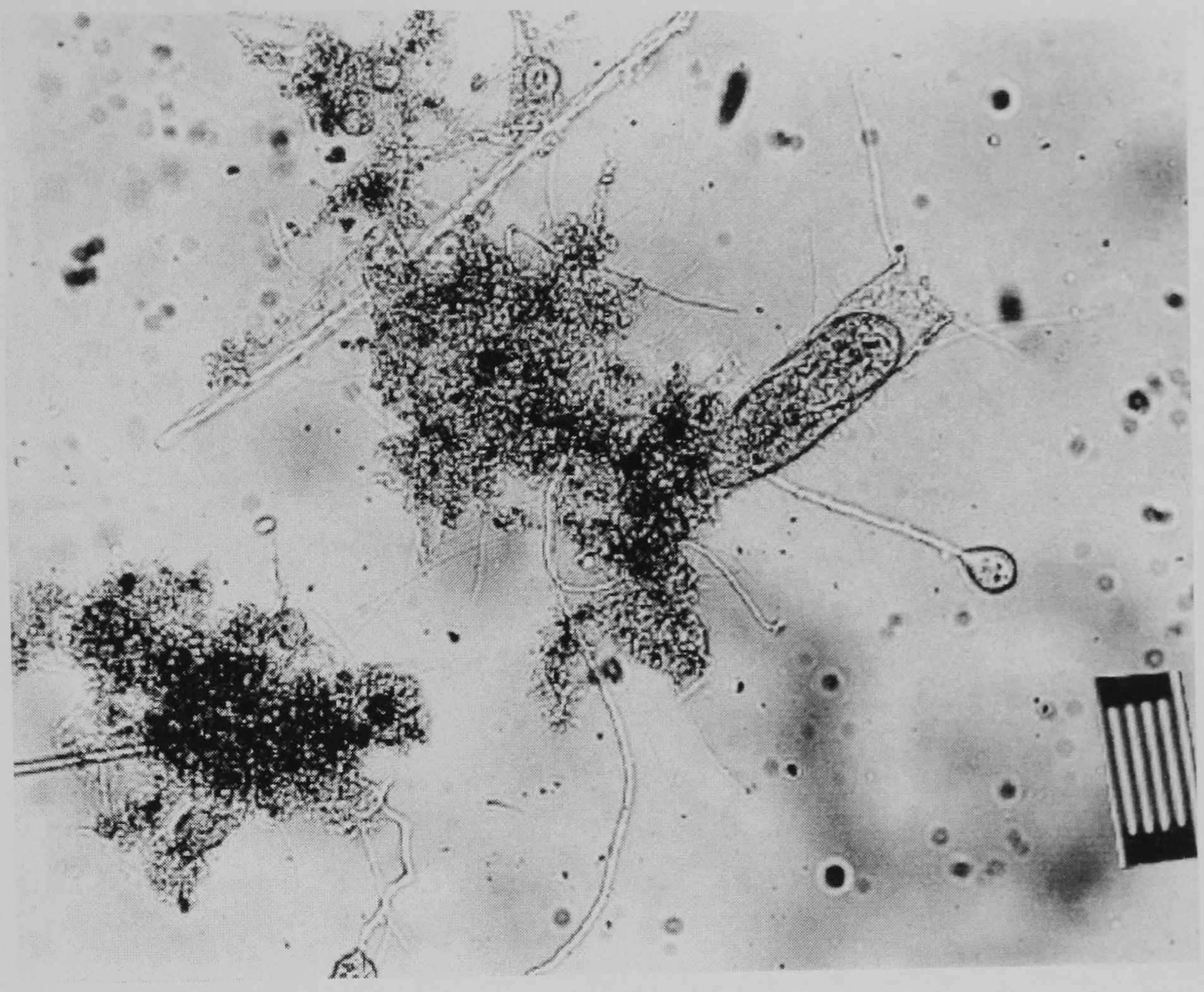


Plate A2.5: Activated sludge treated with 2,4 DNP at pilot scale after 25 d of chemical dosing. Each scale line 0.01 mm apart. Note healthy appearance of rotifer and small size flocs.

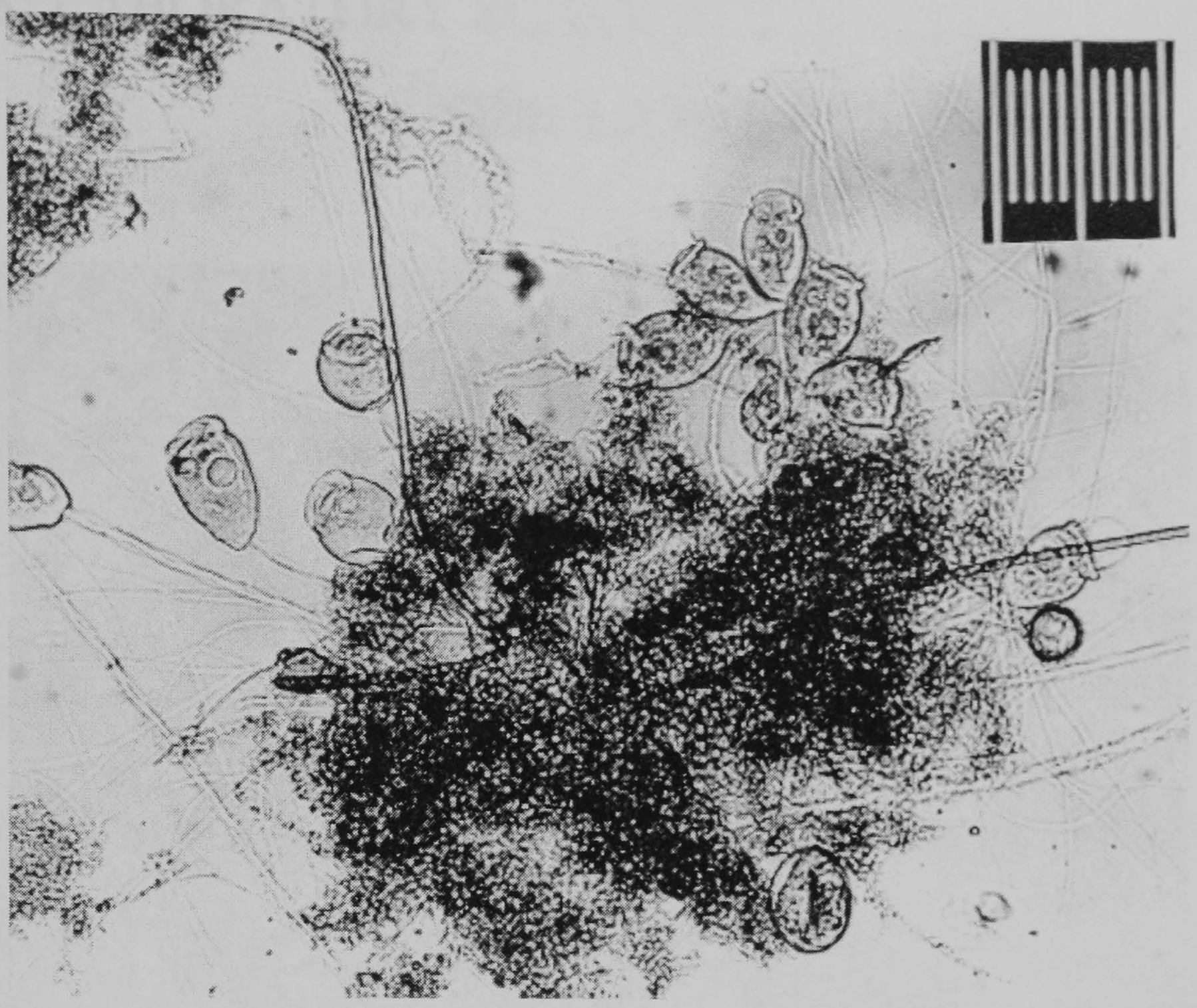


Plate A2.6: Activated sludge treated with 2,4 DNP at pilot scale after 25 d of chemical dosing. Each scale line 0.01 mm apart. Ciliate structure appears unaffected by addition of chemical.

B: LABORATORY SCALE ACTIVATED SLUDGE SIMULATION

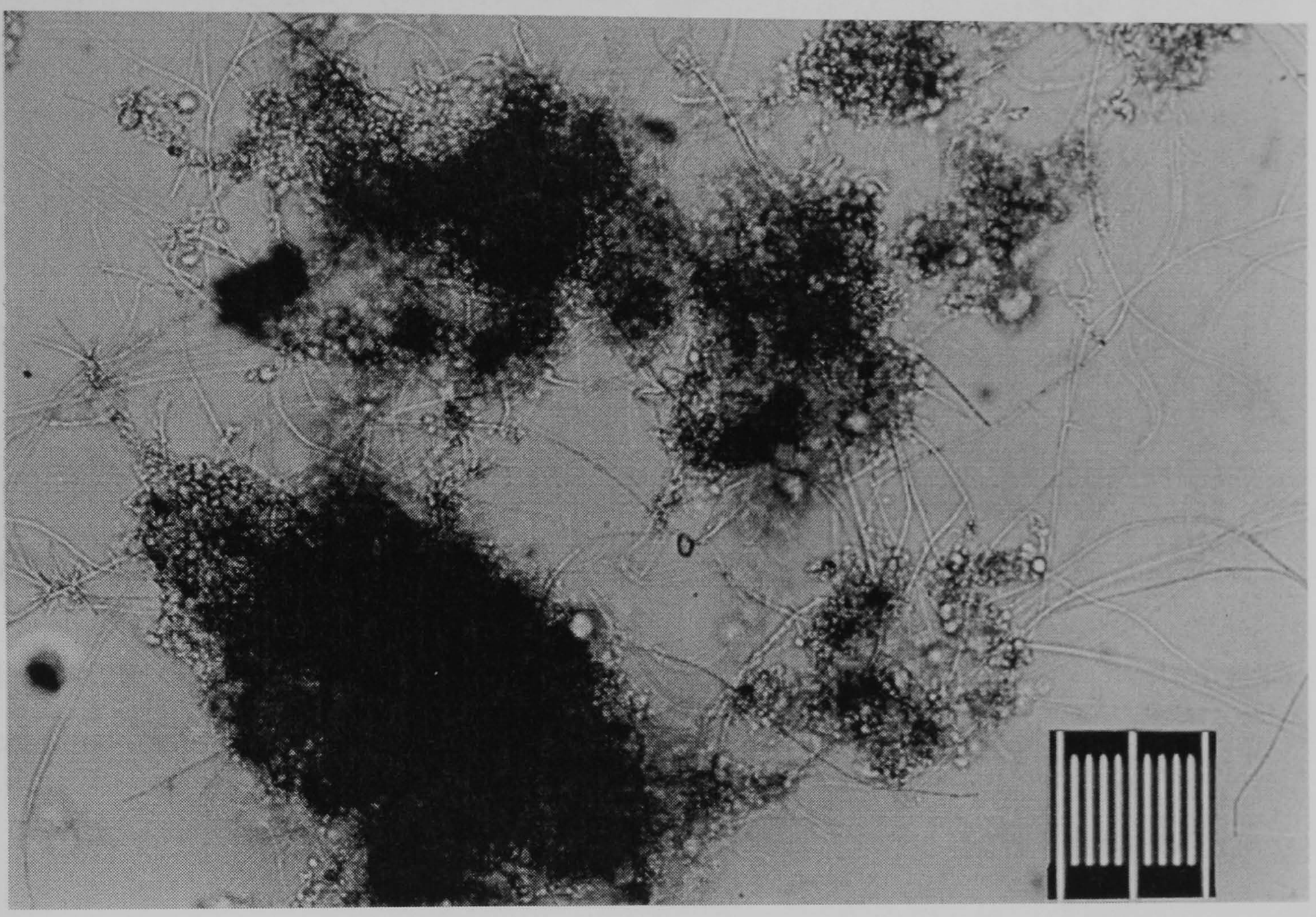


Plate A2.7: Untreated activated sludge from laboratory scale simulation. Scale – each scale line 0.01 mm apart. The lower floc being large compact and rounded in shape.

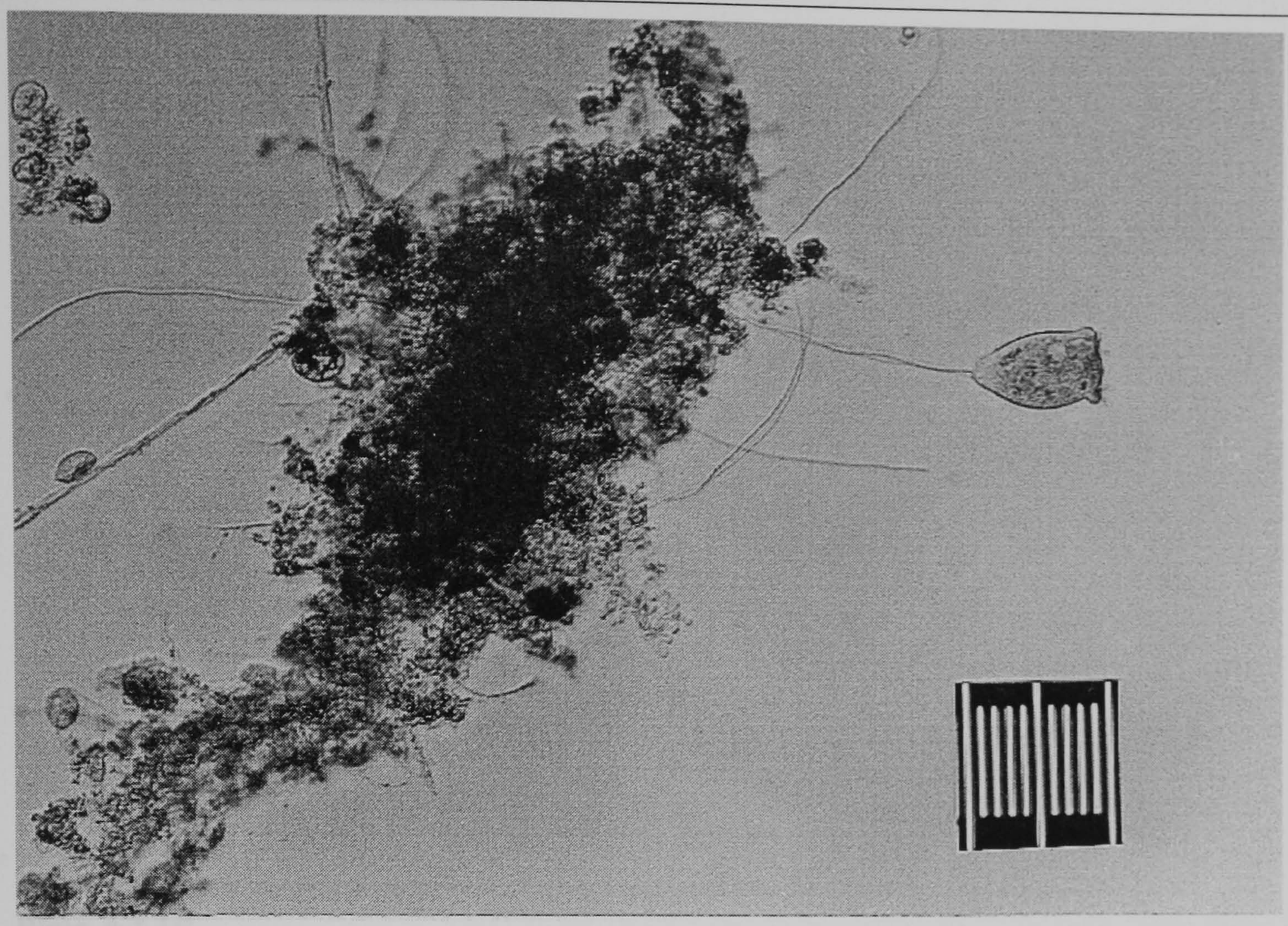


Plate A2.8: Untreated activated sludge from laboratory scale simulation. Scale – each scale line 0.01 mm apart. The lower floc being large and more irregular in shape.

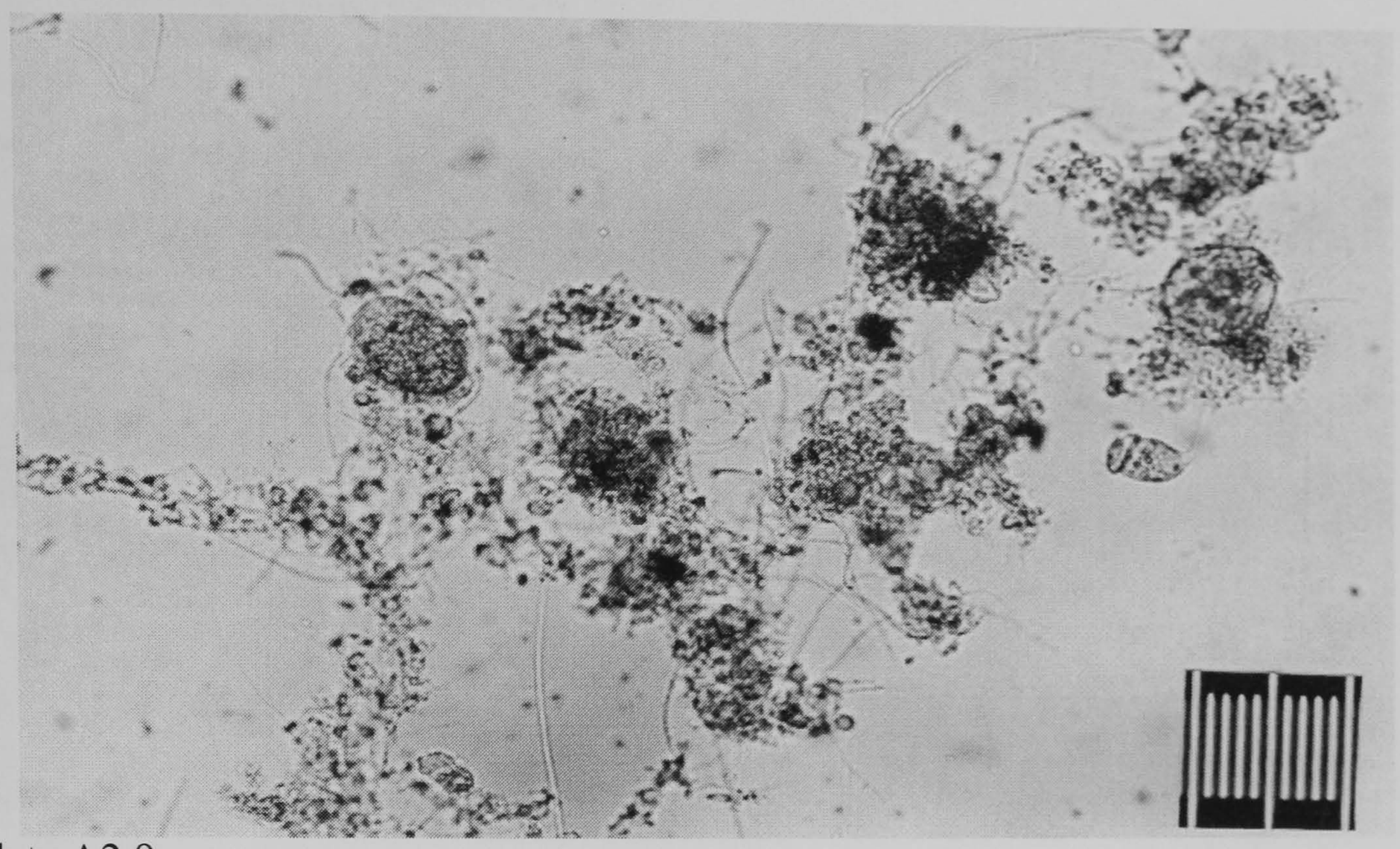
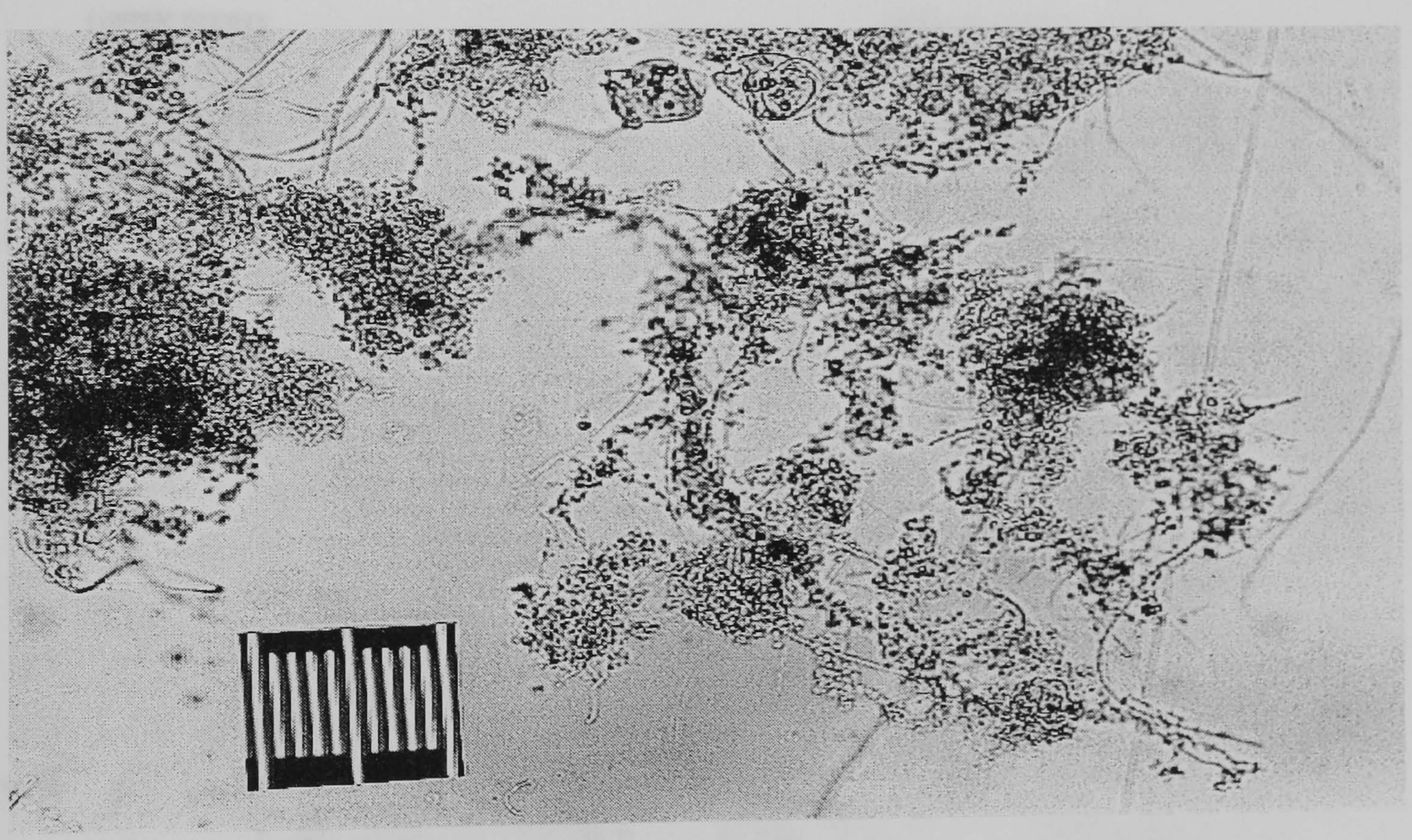


Plate A2.9

Plate A2.10



Plates A2.9 and A2.10: 4 nitrophenol treated activated sludge from laboratory scale simulation after 8 d of chemical dosing. Scale – each scale line 0.01 mm apart. Flocs smaller and more open in structure than untreated.

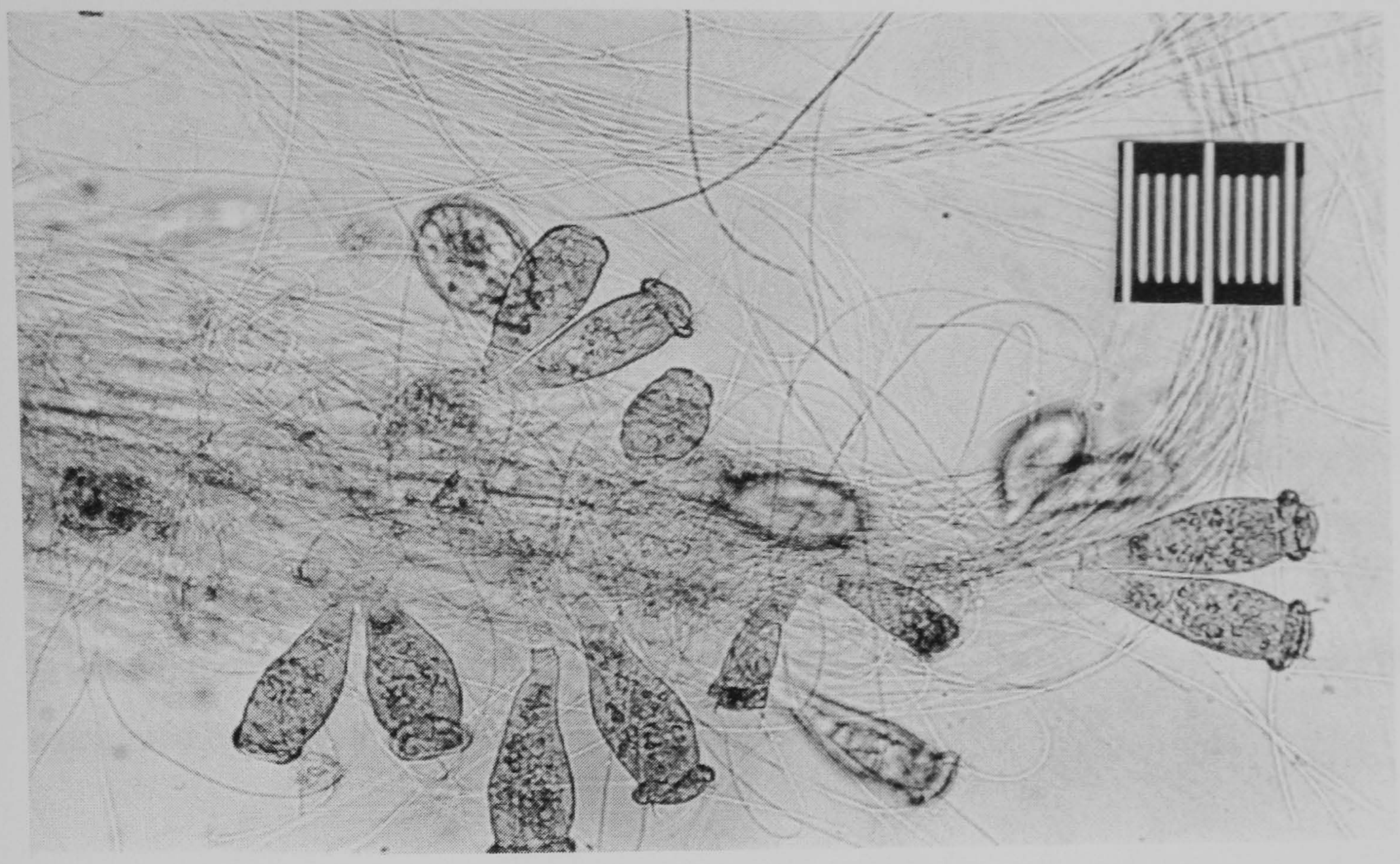


Plate A2.11 - untreated

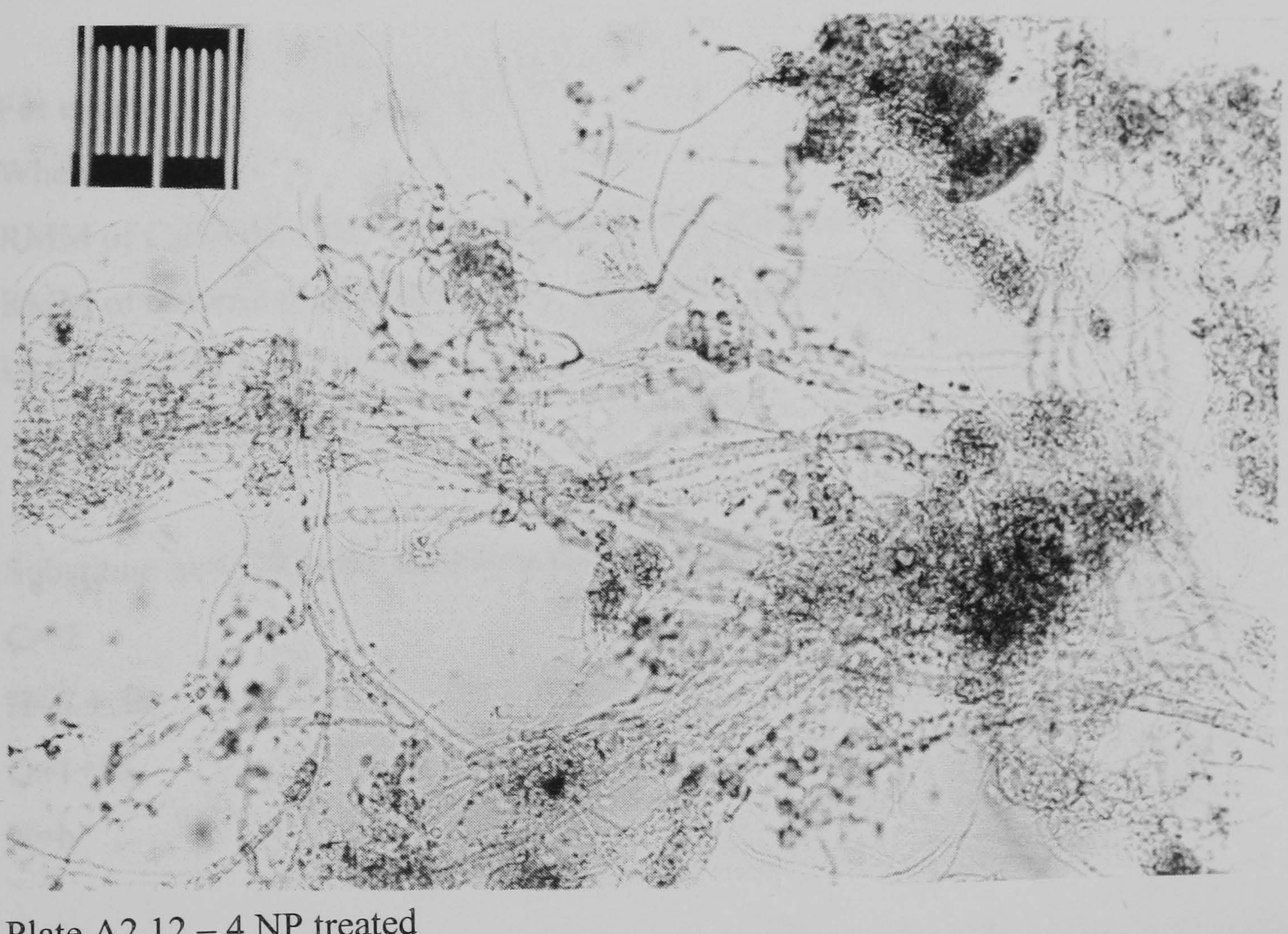


Plate A2.12 – 4 NP treated

Plates A2.11 and A2.12: 4 nitrophenol treated activated sludge from laboratory scale simulation after 8 d of chemical dosing. Scale – each scale line 0.01 mm apart. Note the appearance of ciliate stalks in the treated sample but the absence of the head sections.

APPENDIX 3 :
THEORECTICAL OXYGEN DEMAND

Using a basic formula for biomass as $C_5H_7O_2N$ and using a substrate of propyl alcohol (C_2H_5OH) the theoretical oxygen demand to completely oxidise one unit of substrate can be calculated. From the equation (A3.1) if the substrate is fixed at 1 and the yield is known then the amount of oxygen (in moles) required to oxidise the substrate can be calculated by a series of simultaneous equations.

$$C_2H_5OH + aO_2 + bNH_3 \longrightarrow m\,C_5H_7O_2N + cCO_2 + dH_2O \qquad (A3.1)$$

For example:

When Y=0.7

RMM of C_2H_5OH = 46, 1 kg of C_2H_5OH = 21.7 moles

RMM of bacteria ($C_5H_7O_2N$) = 113, with Y=0.7 get 0.7 kg of biomass produced for 1 kg of C_2H_5OH or 6.2 moles

$\therefore m = 6.2 / 21.7 = 0.29$

Substitute m=0.29 in the following pairs of simultaneous equations:

C= 2	C=5m+c	(A3.2)
H=6 + 3b	H=7m+2d	(A3.3)
O=1+2a	O=2m+2c+d	(A3.4)
N=b	N=m	(A3.5)

This results in a=1.28, that is that 1 mole of C_2H_5OH requires 1.28 moles of oxygen to be fully oxidised.

$\therefore$ 1 kg of C_2H_5OH (21.7 moles) requires (1.28 x 21.7) moles of oxygen or 27.8 moles.

1mole of oxygen has a RMM of 32 ∴(27.1 x 32)g of oxygen required for 1 kg of C_2H_5OH or 888.8g.

This series of calculations was repeated for yield values of 0.7 – 1.2 (Table A3.1)

Table A3.1: Theorectical oxygen demand for propyl alchol

Y	No. moles of oxygen required for 1 mole of substrate (a)	g O_2 kg^{-1} of substrate
1.2	0.81	562.0
1.1	0.90	624.4
1.0	1.0	798.6
0.9	1.15	798.6
0.8	1.18	819.4
0.7	1.28	888.8

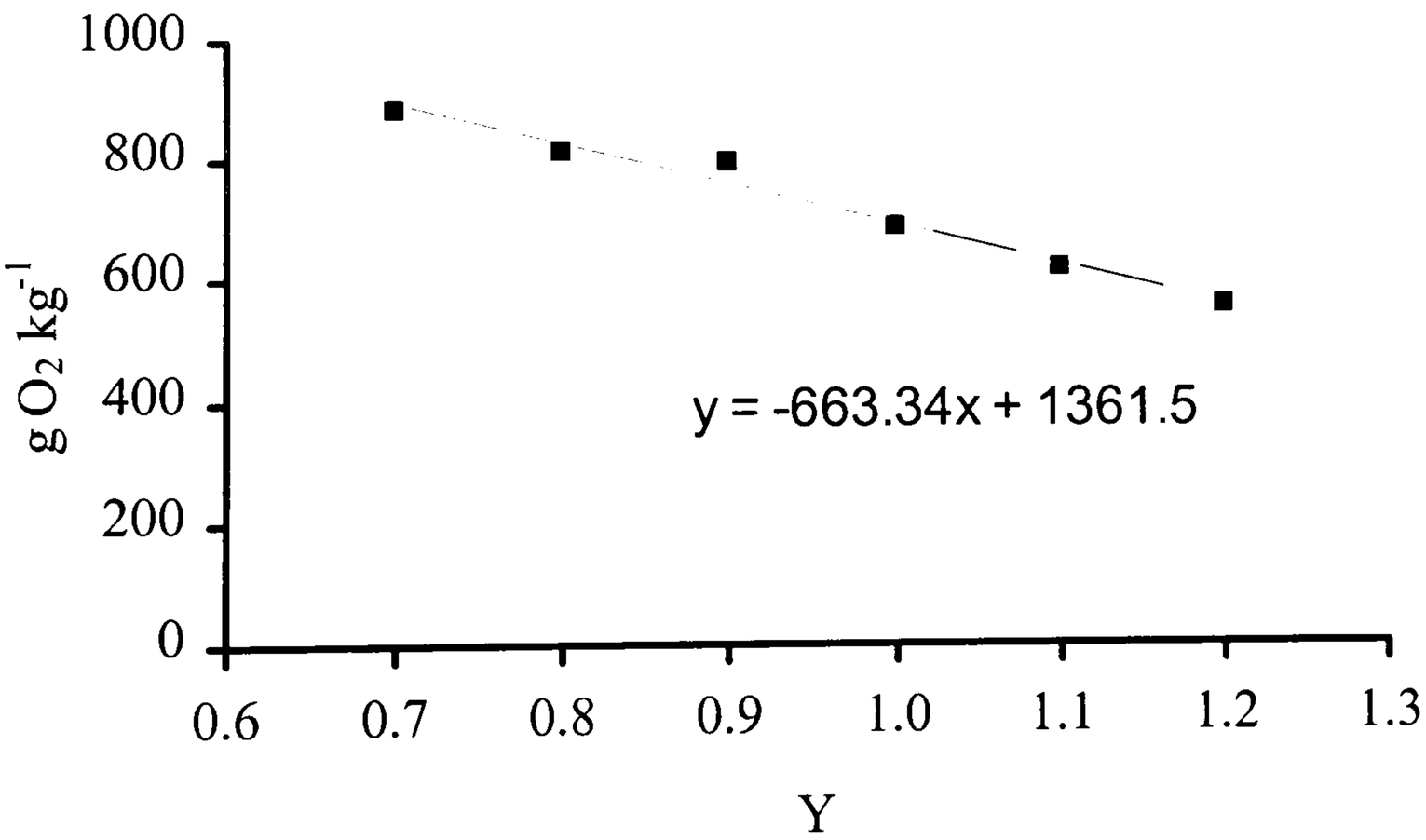


Figure A3.1: Theoretical oxygen demand of propyl alcohol for varying yield values.

From the graphical representation the oxygen demand at yield coefficients of 1.14 and 0.77 as found experimentally in the pilot plant trial were determined as 605.3 and 850.7 g O_2 kg^{-1} of substrate respectively.

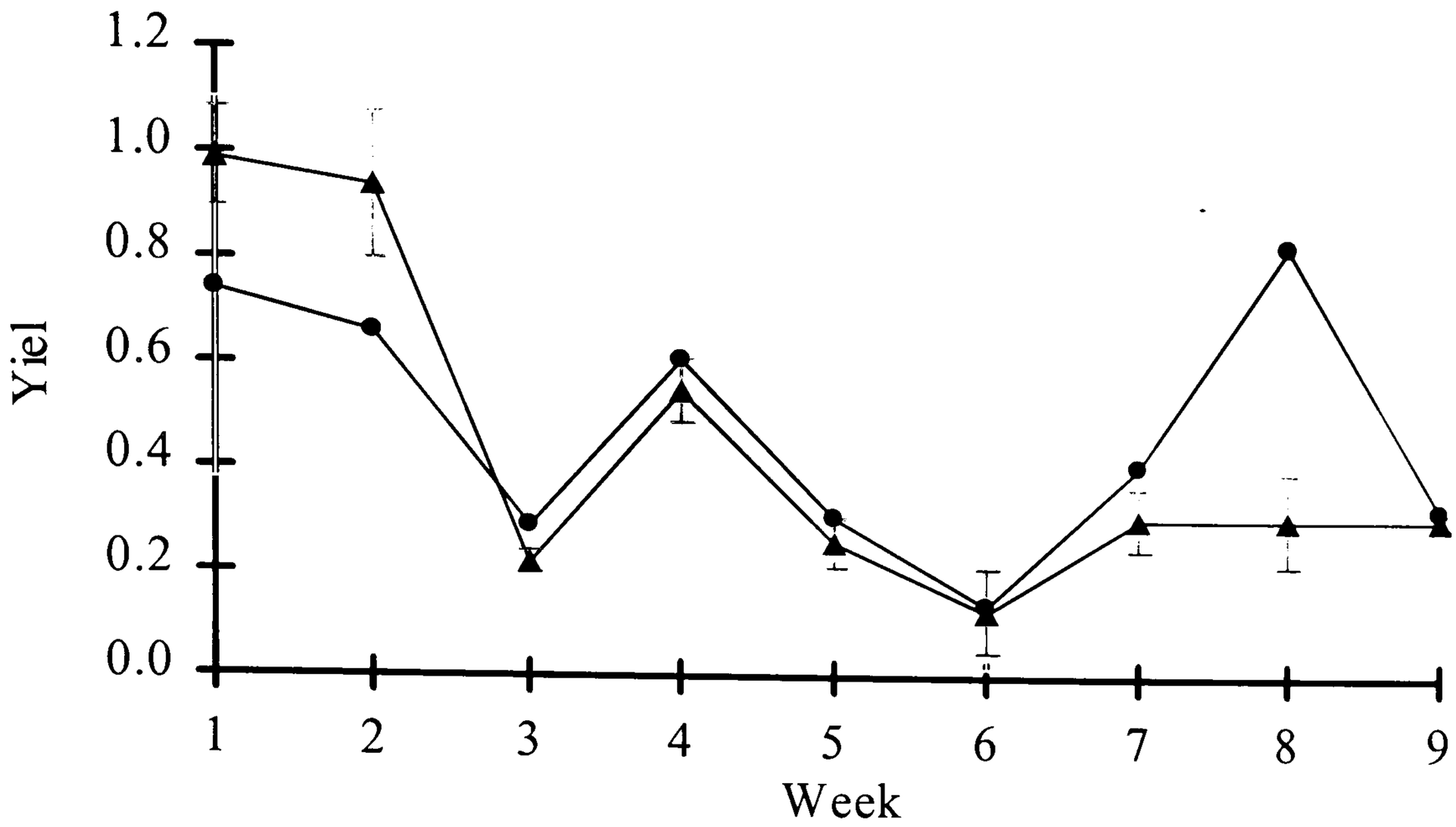


Figure 8.2. Mean yield coefficient of untreated (●) and 2,4 DNP treated activated sludge (▲). Weeks 1 and 2 pre treatment. Error bars represent standard deviations.

Statistically, the yields during the treatment period were compared between the two simulations using an ANOVA to account for the difference between treatment and any variation within the treatment. After treatment with 2,4 DNP the yield of the simulation was significantly reduced from 0.97 ± 0.04 to 0.30 ± 0.05 ($t=6.92$, $P>0.01$) whereas there was no difference in yield statistically between the untreated system before and after treatment. After treatment, the range of yield values for the control at 0.14 – 0.84 was greater than that of the 2,4 DNP treated activated sludge, 0.13 - 0.55. That is, the yield was less variable once chemically treated. The mean yield after treatment was significantly lower than the control (ANOVA $F=3.24$. $P>0.1$). The addition of 2,4 DNP significantly reduced the yield coefficient and therefore the biomass production.